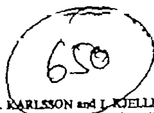
ISSN 0001-6772

Published monthly for the Scandinavian Physiological Society

ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 103 NUMBER 1 May 1978



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APSCAX 103(1) 1 112 (1978)

Editorial office

Acta Physiologica Scandinavica
Karolinska Institutet
S 104 01 Stockholm

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Short Communications

Supplements

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From the Department of Pediatrics I, the Department of Obstetrics and Gynaecology and the Department of Physiology University of Göteborg, Sweden

Measurement of cerebral blood flow in the fetal lamb with a note on the flow-distribution

By

SVANTE BLOMSTRAND, KERSTIN KARLSSON and INGEMAR KJELLMER

Received 11 July 1977

Abstract

BLOMSTRAND, S., K. KARLSSON and I. KJELLMER. *Measurement of cerebral blood flow in the fetal lamb—with a note on the flow-distribution.* Acta physiol. scand. 1978. *103* 1–8

The cerebral blood flow was measured in the acutely exteriorized fetal lamb by ^{133}Xenon washout and microsphere distribution techniques. The measurements were performed at different blood gas levels. Regional cerebral blood flow was calculated from the microsphere distribution for five different parts of the brain. This gave estimates for blood flow in both the grey and white matter of the hemispheres, which were in close agreement with the cerebral blood flow estimated by the ^{133}Xe washout technique. The microsphere distribution shows that the fetal cerebral hemisphere has a low blood flow compared to the basal parts of the brain, and that this difference is increased during hypoxia and hypercarbia.

Key words: Cerebral blood flow, Fetus, ^{133}Xenon (-washout), Microspheres
Abbreviations: CBF Cerebral blood flow, rCBF Regional cerebral blood flow

In previous studies we have used the ^{133}Xenon washout technique for estimating the blood flow of cerebral cortex in the acutely exteriorized fetal lamb (Karlsson *et al.* 1974, Kjellmer *et al.* 1974). Purves and James (1969) thoroughly discussed the limitations and applicability of this method. They used local microinjections of ^{133}Xenon in grey or white matter of cerebral cortex in fetal lambs to validate the method of resolving the washout curve into two phases—one fast and one slow phase. Their conclusions were that these two phases corresponded to grey and white matter clearance rates and thus permitted the calculation of grey and white matter blood flow. Although they were unable to recover any recirculating isotope, the very organization of the fetal circulation with *1/3–1/2* of the combined cardiac output traversing the umbilical flow circuit, while the remaining flow bypasses the placenta, necessitates a proportion of tracer recirculating to the brain. Thus the slow phase most plausibly in part will reflect recirculation of ^{133}Xenon.

We therefore used only the fast phase of the clearance curve to calculate the CBF. At basal blood gas levels this was estimated to 0.5–0.7 ml/g min^{-1} (Kjellmer *et al.* 1974). This is in close agreement with the values reported by Purves and James (1969), as is the rise to

two to threefold values when the animal is exposed to low oxygen and/or high carbon diox tensions.

Rudolph *et al* (1970) and Makowski *et al* (1972) using the microsphere distribution technique in the chronically instrumented lamb fetus, arrived at considerably higher estimat of CBF *i.e.* 0.9–1.2 ml/g min at basal conditions. Several possible explanations prese themselves to account for this discrepancy. The [133]Xenon method might underestima blood flow the blood flows may be truly different because of the difference between the e teriorized preparation and the unanaesthetized chronic preparation or because of differenc in blood gas levels. The difference might also be attributed to a grossly inhomogeneo blood flow distribution within the brain, since the flow values derived from the micro sphere experiments refer to overall blood flow of the brain while the flow values obtaine by the [133]Xenon technique most probably only reflect cortical blood flow

The present series of experiments was therefore undertaken to answer two questions

1) is the blood flow homogeneously distributed to the various parts of the brain at norm and reduced oxygen tensions?
2) does the rapid phase of elimination in the [133]Xenon washout curve correspond to bloo flow in the grey matter of the cerebral cortex?

Material and Methods

10 pregnant ewes with 13 fetuses were investigated. Gestational age, which was either known o estimated from weight and crownrump length of the fetuses according to the values given by Joubert (1956), and between 120 and 145 days. The ewes were anesthetized with Ketamin 1.5–2.0 mg/kg and anesthesia was maintained with chloralose 35 mg/kg with small doses added hourly. The ewe was tracheotomized and ventilated with known gas mixtures with a pressure cycled vent l t Arterial blood gases were used to direct respirator settings. A slow c infusion of 10% glucose solution was given t maintain the blood sugar t normal levels throughout the experiment.

The f tus was exteriorized and placed on thermostated table where body temperature and humidity could be maintained. Great care was taken to minimize disturbance of the umbilical circulation. The preparation of the fetus ha been described previously (Kjellmer *et al* 1974). It i volves cannulating the trachea and connecting it to a saline filled reservoir cannulating the right brachial artery for blood pressure recordings and arterial blood samples, and measuring deep body temperature *ie* thermistor in the rectum

Washout method

The principal arrangement of the preparation is shown in *Fig 1 A*. The left lingual artery wa cannulated i the retrograde direction with polyethylene t bing, PE 50, for the injection of [133]Xenon saline into the carotid artery. Gamma radiation wa recorded over the skull w th a thallium-activated scintillation detector diameter 5 cm. The detector w s f tted in a lead cylinder 10 cm from the bott m, where a lead diaphragm with a central hole 2 cm in diameter was shielding the detector. This diaphragm was placed 1 cm from the fetal scalp and centered over the left parietal region. The radioactivity was recorded with ratemeter time constant 3 s, and written out by linear potentiometer i k recorder (Servogor Goerz Elektro, RE 520, Austria) at a paper speed of 0.5 mm/s. The washout curves were analysed a described previously (Kjellmer *et al* 1974). After background subtraction a semilogarithmic plot wa drawn and the slow phase of tracer washout defined. After subtracting this phase from the total curve the resulting rapid clearance phase was obtained and its halftime used t calculate the cortical blood flow

Microsphere method

In F g. 1 B the principal arrangement of the fetal circulation and of the preparation is shown. T femoral vein was cannulated and catheter introduced into the inferior caval vein. Through this

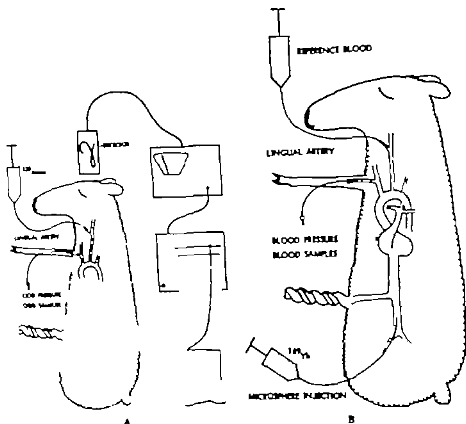


Fig. 1. A schematic presentation of the set up and preparation for determination of CBF by the ^{133}Xenon technique (1A) and for determination of CBF by the microsphere technique (1B). In the latter drawing the fetal shunts are also presented.

sion of microspheres, diameter 25 µm, tagged with ^{169}Yb was injected. The injection chamber was carefully agitated before and during the injection by a high frequency whirlmixer. The injection of approx. 300 000 spheres in 0.2 ml Dextrane solution lasted 15 s, and then the chamber was rinsed by the further injection of 6–10 ml of 6 Dextrane solution. The reference blood was obtained by the continuous withdrawal of blood at a constant rate (1.50 ml/min) through the catheter in the lingual artery. The withdrawal pump was started 15 before the injection, and blood was collected for a total time of 2 min.

After the experiments had been ended, the brain of the fetus was removed and dissected into 4 different parts, medulla oblongata and pons, cerebellum, hemispheres, and the rest of the forebrain, called basal ganglia. The hemispheres were further divided in white and grey matter. The fresh wet weight of these 5 parts was determined. The radioactivity of the reference blood and of the different parts of the brain was then measured using a whole body counter designed for use with small animals (Packard, Model 440 Armac Counting System). By measuring the activity in a known number of spheres, the total amount of spheres in each specimen could be calculated. According to Buckberg *et al.* (1971) at least 400 spheres should be distributed to each specimen to make the estimate of blood flow reliable. This was achieved on all occasions.

The CBF-determinations were made at normoxia and at different levels of hypoxia. The ewe was ventilated with gas mixture, which gave the desired level of oxygen and carbon dioxide tension in her blood. The fetal CBF was then determined with the ^{133}Xenon technique, the injection being given not earlier than 20 min after the respirator gases had been changed. After recording the washout curve for 20–25 min, the

two to threefold values when the animal is exposed to low oxygen and/or high carbon dioxide tensions.

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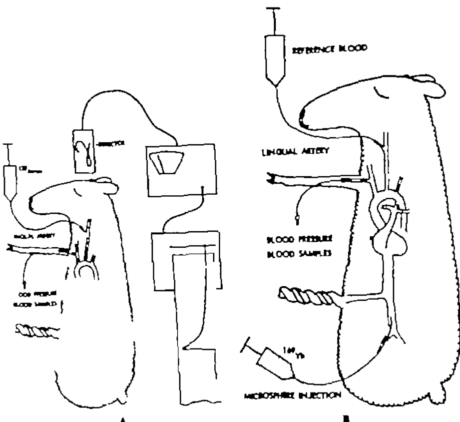


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TABLE II Linear regression constants.

$CBF_{microsphere}$	= a	+	$b(CBF_{Xenon})$	r
Cerebellum	− 61		2 81	86
Pons + Medulla	−1 87		4.82	85
Basal ganglia	−1 52		3 71	84
Hemisphere, white	− 14		1.23	86
Hemisphere, grey	−.35		1 43	.86
Total brain	− 55		1.93	.87

The constants a and b in the equation above, which define the regression lines shown in Fig. 3 B, are given in this table together with the coefficient of correlation, r for the different lines. The equation is valid for $.51 \leq CBF_{Xenon} \leq 1\ 10$.

parts of the brain are much steeper than for the hemispheres. In Table II the equations for these lines are given together with their r values. Thus there exists a close correlation between the rCBF for all the regions obtained by the microsphere technique and the CBF_{Xenon} but it is also apparent from Fig. 3 B that blood flow in the basal parts of the brain is higher *than in the hemisphere, in particular during hypoxia.*

Discussion

The rCBF in the white and grey matter of the hemisphere were not significantly different *in the present series of expts. This is in contrast to earlier reports in other experimental* conditions. Reivich *et al* (1969) using an autoradiographic technique in the adult cat demonstrated quite marked differences between the blood flow in the cortex and in the marrow of the hemisphere, with a higher flow in the cortex, and so have Kennedy *et al* (1972) using the same technique in the newborn dog. The failure in our series of expts. to document a difference between grey and white matter blood flow may be due to too crude a separation between grey and white matter in our preparation It may also reflect difference due to anesthesia. We have chosen Ketamine instead of barbiturates to induce the anesthesia because of the marked reduction of cerebral oxygen uptake caused by the latter and hence a possible effect on CBF Chloralose was used for maintenance since it has been regarded as "the ideal anesthetic agent for the study of cardiovascular reflexes" (Greisheimer 1965). Our earlier experiences with a similar preparation have shown the fetus to have a measureable cortical electrical activity during this anesthesia and at these blood gas levels (Karlsson *et al* 1974) *However the functional state of the cortex must* be depressed by the anesthesia, and this might contribute to a less pronounced difference in blood flow between grey and white matter compared to awake animals. Speculatively it may also reflect a functional state of the cerebral cortex during fetal life with a low oxygen consumption and a low blood flow in a poorly developed rather inactive neuronal part of the cortex and a metabolically relatively active, myelinating white matter In Table III our estimates of rCBF in the fetal lamb are compared to other materials, mostly from adult animals. The highest blood flow in the adult animal is found in the cerebral cortex (Reivich *et al* 1969 Foreman *et al* 1976, and Marcus *et al* 1976), *while in the newborn dog* the highest flow is found in thalamic

TABLE III. Regional cerebral blood flow in different species. A comparison between the present study and data from the literature.

Author	Present study	Kennedy *et al.* 1972		Reivich *et al.* 1969	Foreman *et al.* 1976	Martin *et al.* 1976
Animal	Fetal lamb	Newborn dog	Adult dog	Adult cat	Adult pig	Adult dog
Condition	Anesthetized	Awake	Awake	Awake	Awake	Awake
Technique	Microspheres	Autoradiography	Autoradiography	Autoradiography	Microspheres	Microspheres
Measurements of rCBF from						
Hemisphere, grey	.47	.30–.40	.45–.80	.74–1.22	.60	.73
Hemisphere, white	.40	.08–.30	.10–.25	.21	.41	.30
Cerebellum, grey	—	.30–.40	.55–.60	.33	.56	—
Cerebellum, white	—	—	—	.24	.35	—
Cerebellum, whole	1.08	—	—	—	—	.66
Basal ganglia	.73	.40–.50	.70–1.05	.54–1.06	.43	.45
Pons and medulla	.90	—	—	—	.30	.47

All determinations of rCBF were made during normoxia, and in the present study this means the 5 fetuses with arterial pO_2 > 2.39 kPa. The rCBF is measured in ml/g·min^{-1}

nuclei and in cochlear and vestibular nuclei (Kennedy *et al.* 1972). In the newborn dogs the differences between the highest and lowest rCBF are small compared to the corresponding differences in the adult dogs. They were also able to demonstrate that the blood flow to different structures in the brain rose from a comparatively low level in the newborn puppy to a peak level at an age of 3–8 weeks and then declined to the adult level. The peak levels of blood flow occur during the time when the myelinization is most intense in a region. It is tempting to suggest that the distribution of flow in the fetal brain, which is quite different from the pattern in adult animals, is explainable along the same lines.

The differences between our earlier determinations of CBF using the ^{133}Xenon washout technique (Kjellmer *et al.* 1974) and others using the labelled microsphere distribution technique (Rudolph *et al.* 1970, and Makowski *et al.* 1972) can now be explained by the present findings. The ^{133}Xenon washout technique measures the blood flow in the cerebral hemisphere accurately but this region in the fetal brain has a lower blood flow than the basal cerebral structures. The microsphere technique used to measure overall cerebral blood flow will thus give a higher value, and the difference between the two methods will be greater during hypoxia or hypercarbia with a higher blood flow which is apparent from the equation for the regression line for the whole brain in Table II.

We are obliged to civil engineer Åke Cederblad, Dept. of Nuclear Physics, Sahlgren Hospital, for his kind help with measurements of the activity from the microspheres. This study was supported by Medical Research Council (Grant 19X 2591) and by Prenatalforskningsfonden.

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The effects of Triton-detergents on the stretch receptor of the crayfish

By

D. Ottoson and B. Rydqvist

Received 19 October 1977

Abstract

Ottoson, D. and B. Rydqvist. *The effects of Triton-detergents on the stretch receptor of the crayfish.* Acta physiol. scand. 1978. *103.* 9–18.

The effects of the non-ionic detergents Triton X-45 and Triton X 100 on the action potential and the receptor potential of the stretch receptor neuron of the crayfish *Astacus fluviatilis* was studied with intracellular recording technique. Membrane currents were measured with voltage clamp technique. Both detergents blocked the action potential in 20–30 min at concentrations of 60–80 µM. Following blocking of spike electrogenesis the receptor potential evoked by stretch was obtained in isolation. With prolonged exposure of the neuron to the detergents there was slowly developing reduction of the receptor potential and after 60–90 min no response to stretch could be obtained. These effects were produced without any significant change of the resting membrane potential. Following return to normal saline the responsiveness to stretch was completely restored in 80–100 min. Measurements with voltage clamp technique showed that the passive membrane properties were little affected by the two detergents. The stretch induced current on the other hand was severely depressed and almost abolished with prolonged exposure. The experimental results suggest that non-ionic detergents block the spike electrogenesis and the transducer action by selective action on the sodium channels of the membrane of the receptor neuron.

It has been reported that non-ionic detergents have an action on peripheral nerves and muscle closely like local anesthetics (Soehring, Dass and Stave 1952, Zipf and Dittmann 1964, Dittmann 1973). The mechanisms underlying the blocking effect are unknown. Recent observations by Brismar and Rydqvist (1978) suggest, however, that non-ionic detergents have a selective action on the sodium channels of the nerve membrane. This observation raised the question whether or not these substances also blocked the activity of peripheral receptors.

It is known from studies on different kinds of sensory organs that the properties of the receptor membranes differ in many respects from that of membrane producing action potential. The main portion of the current induced in transducer membranes during activity is carried by sodium ions but there is also an unknown amount of contribution of other ions. These currents are resistant to the action of many substances like tetrodotoxin (TTX) and local anesthetics.

The present study was undertaken in order to examine the action of two members of the Triton series with different lipophilicity on the crustacean stretch receptor organ. The dendrites of this organ are sensitive to stretch of the muscle to which the neuron is attached. The cell is relatively large and this makes it possible to measure the membrane current induced by a given stimulus with voltage clamp technique. The stretch receptor neuron therefore appeared to be suitable as a model for the study of the action of the detergents on the receptor membrane. The results show that Triton X-45 and Triton X 100 have a blocking effect on the impulse generating membrane of the neuron. With prolonged exposure the activity of the transducer membrane was reduced and finally abolished. There was no appreciable change of the resting membrane potential accompanying the blocking action. It is suggested that the observed effects are due to an action mainly on the potential dependent sodium permeability mechanism of the impulse generating membrane as well as of the transducer membrane of the neuron.

Methods

The experiments were carried out on the slowly adapting stretch receptor of the crayfish (*Astacus fluviatilis*). The receptor neuron together with its muscle were isolated from the second or third abdominal segment and mounted i a small chamber filled with saline of the following composition in mM NaCl 2?6, KCL 5.4 $CaCl_2$ 13.5 $MgCl_2$ 1.2 (van Harreveld 1936) and was buffered with 10 mM Tris to pH 7.-7.4 (23°C). The temperature of the bath was kept at +15°C.

The recepto muscle was tied at each end t a thin metal rod (0.1 mm), each rod being attached to a Brush pen motor with position and velocity feed-back. The position transducer signal was used to record displacement. Stretch was applied by symmetrical extension of the receptor muscle towards its both ends. The parameters of the applied stretch were varied by driving the pen motors w th electrical pulses of different wave forms.

The receptor neuro was penetrated with two microelectrodes, one being used to record the membrane potential, the other t pass current. Both electrodes were filled with 3 M KCL and had a resistance in the range of 5–10 MΩ. The membrane potential was recorded with a Mentor N 950 preamplifier from which the signals were fed into tape-recorder (Hewlett Packard 3960 ru with passband 0–1250 or 0–5 000 Hz) before being displayed on a Tektronix 5321 oscilloscope The output of the Mentor amplifier was connected to a clamp amplifier with a maximum output of ±150 V The loop ga was set to values between 5 000 to 8 000 depending on the resistance of the microelectrodes (Brown, Ottoso and Rydqvist. To be published). Membrane current was measured across feed-back resistor of an operational amplifier used to maintain the bath at ground potential via n Ag-AgCl-electrode. The measurements of the membrane current were made with the receptor muscle kept at resting length and during the pplication of brief (about 1) ramp and hold extensions. The membrane potential in these experiments was gradually shifted to a positive potential value of bout +40 mV from this value the potential was moved in steps of about −10 mV

The current measurements were made t time when the membrane current had stabilized t the new level which usually occurred 30 s after application of the command pulse.

The rat f rise of the action potentials was measured by differentiating signals through a Miller-differentiator and was displayed together with the action potentials on the oscilloscope screen.

The effect of Triton X-45 and Trito X 100 were tested in preliminary experiments to find out the time course of their action at different concentrations. In the present study concentrations between 40 and 80 μM were chosen since at these concentrations the blocking effect developed at time course which allowed for carrying out the current measurements. Triton X-45 and Triton X 100 were gifts from Rohm and Haas Co. and used without further purification. These substances belong t a series of homologous polyoxyethylen-p-t-octyl-phenols with varying umber of oxyethylene groups. Triton X-45 has an verage number of 4.5 oxyethylene units and a molecular weight of 426 Triton X 100 has an average number of 9.8 oxyethylene units and a molecular weight of 628 (Rohm and Haas, CS-40 l/cf and CS-Ola, see also Simons, Helenius and Garoff 1973). The HLB numbers (hydrophilic lipophilic balance umber) for Triton X-45 nd Triton X 100 are 10.4 and 13.5 respectively

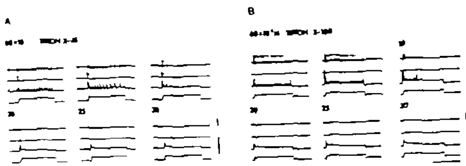


Fig. 1. Impulse response of crayfish stretch receptor to steplike stretch before and during exposure to *A*: 60 µM Triton X-45 and *B*: 60 µM Triton X 100 for 30 min. Numbers above each recording represent time in min after application of detergent. Top trace, differentiated potential signal, calibration 200 V/s. Second trace from top: zero potential. Third trace from top: membrane potential change, calibration 100 mV. Bottom trace, stretch in arbitrary units. Time bar 1 s.

Results

Effect on spike electrogenesis

Exposure of the receptor to Triton X-45 at a concentration of 60 µM caused a gradually developing block of the impulse response to an applied test stretch (Fig. 1 *A*). The discharge of impulses decreased in duration so that after 10 min a few spikes only were elicited by the extension and only an abortive response was seen after about 25 min. The abolition of the impulse response was preceded by a decrease in amplitude and rate of rise of the individual spikes. As can be seen in Fig. 2 *A* the maximum rate of rise (V_A) of the spikes falls slowly with time of exposure. At a concentration of 40 µM blocking does not occur until after more than 30 min. With higher concentrations (60 µM) the effect on the spikes develops faster and all signs of impulse generation are generally abolished within 20–25 min. In most experiments blocking was not accompanied by any significant change of the resting membrane potential. The change of membrane potential due to receptor activity (receptor potential) remained after the disappearence of the regenerative spikes. Hence the effects of Triton was similar to that of TTX and local anesthetics like procaine and lidocaine in the respect that they all affect the specific sodium permeability mechanism.

On return to normal solution the discharge due to extension reappeared. Recovery was slow and usually incomplete as can be seen in Fig. 2.

The effect of Triton X 100 was closely similar to that of Triton X-45 (Figs 1 *B* and 2 *B*). In general, however, the blocking effect developed more slowly than with Triton X-45 at the same concentration of the two substances. As with Triton X-45 blocking of the impulse discharge was not accompanied by any significant change of the resting membrane potential. Recovery of the impulse response had a slow time course similar to that after exposure of the neuron to Triton X-45.

Effect on the receptor potential

Prolonged exposure of the neuron to the detergents caused a block of the impulse generation and a reduction and final elimination of the receptor potential as is illustrated in Fig. 3

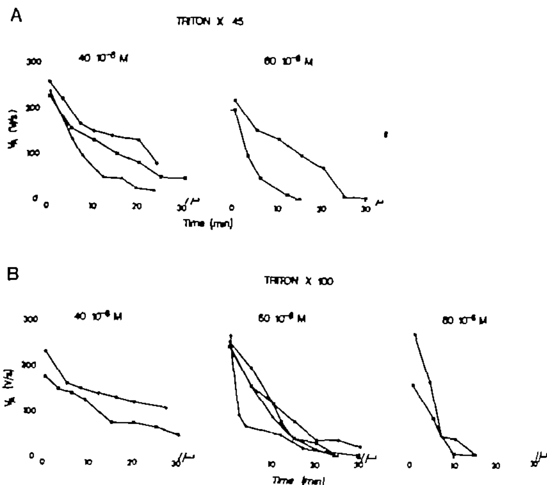


Fig. 2. Maximum rate of rise (V_A) of action potential versus time f llowi g exposure of neurons to Triton detergents. Measurements were made over the first 20–30 min and the cells thereafter left for varying periods of time in Triton before being returned t normal saline.
A. Triton X-45. In the left hand graph one cell (circles) was exposed for 35 min and recovery in normal saline was measured after additional period of 130 min second cell (squares) was exposed for 200 min and recovery measured after 110 mi nd third cell (triangles) was exposed for 200 min and recovery measured after 140 min I the right hand graph one cell (circles) was exposed for 100 min and recovery measured after 140 min the other cell (squares) was exposed for 25 min and recovery measured after 105 min. *B*. Triton X 100. In the left hand graph one cell (circles) was exposed for 70 min but was not returned to normal saline second cell (squares) was exposed f r 3 min and recovery measured after an additional perod of 110 min. In the middle graph one cell (squares) was exposed for 35 min and allowed to recover for 100 min another cell (triangles) was exposed for 120 min and recovery followed for 100 min. The other cells were not returned to normal saline.

After application of Triton X-45 (Fig. 3 *A*) the spikes were abolished after 30 min leaving the receptor potential in isolation Following this there was a gradual slow reduction of the receptor potential and after 90 min exposure of the neuron to Triton X-45 extension did no longer evoke any noticeable receptor response. At this stage the extension had no effect whatsoever on the neuron. The time course of the blocking effect of Triton X-45 on the dynamic and the static phase of the receptor potential is illustrated in Fig. 4 The spikes during static extension of this neuron disappeared after 10 min while the dynamic spike was not

A

60 μM TRITON X 45

0 30 60 90

120 150 180 240

B

60 μM TRITON X 100

0 60 120 180

Fig. 3. Impulse and receptor response of crayfish stretch receptor to steplike stretch before and during prolonged exposure to 60 μM Triton X-45 (*A*) and 60 μM Triton X 100 (*B*). Numbers above each recording represent time in min after initial application of detergent. Top trace: differentiated potential signal, calibration 200 V/s. Second trace from top: zero potential. Third trace from top: membrane potential change, calibration 100 mV. Bottom trace: stretch in arbitrary units. Time bar: 1 s. In *A* the cell was returned to normal saline after 100 min, in *B* after 1[illegible]0 min.

blocked until after 20 min. Following the block of impulse generation the dynamic and the static phase of the receptor potential remained unaffected for about 30 min. The effect of Triton X-45 on the transducer activity of the neuron thus required an exposure time of about 45 min to develop. Usually the dynamic phase of the response was first affected fol-

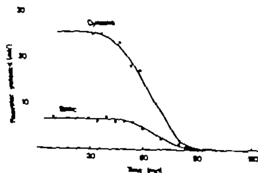
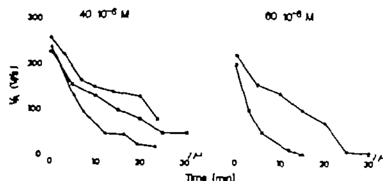


Fig. 4. Effect of Triton X-45 on dynamic and static phase of receptor potential (see text).



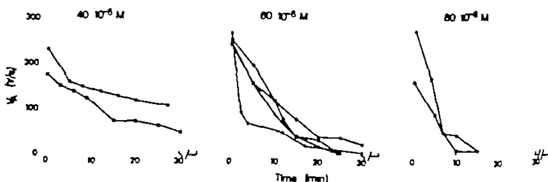


Fig. 2. Maximum rate of rise (V_A) of action potential versus time following exposure of neurons to Triton detergents. Measurements were made over the first 20–30 min and the cells thereafter left for *varying periods of time in Triton before being returned to normal saline*.
A. Triton X-45. In the left hand graph one cell (circles) was exposed for 35 min and recovery in normal saline was measured after an additional period of 130 min; a second cell (squares) was exposed for 200 min and recovery measured after 110 min and a third cell (triangles) was exposed for 200 min and recovery measured after 140 min. In the right hand graph one cell (circles) was exposed for 100 min and recovery measured after 140 min; the other cell (squares) was exposed for 25 min and recovery measured after 105 min. *B*. Triton X 100. In the left hand graph one cell (circles) was exposed for 70 min but was not returned to normal saline; a second cell (squares) was exposed for 3 min and recovery measured after an additional period of 110 min. In the middle graph one cell (squares) was exposed for 35 min and allowed to recover for 100 min; another cell (triangles) was exposed for 170 min and recovery followed for 100 min. The other cells were not returned to normal saline.

After application of Triton X-45 (Fig. 3 *A*) the spikes were abolished after 30 min leaving the receptor potential in isolation. Following this there was a gradual slow reduction of the receptor potential and after 90 min exposure of the neuron to Triton X-45 extension did no longer evoke any noticeable receptor response. At this stage the extension had no effect whatsoever on the neuron. The time course of the blocking effect of Triton X-45 on the dynamic and the static phase of the receptor potential is illustrated in Fig. 4. The spikes during static extension of this neuron disappeared after 10 min while the dynamic spike was not

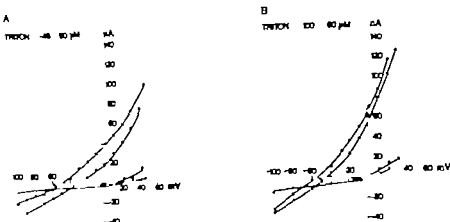


Fig. 6. I–V relations of the steady-state passive currents (solid symbols) and SIC (open symbols) measured as in Fig. 5 before and after treatment of the cell with 60 μM Triton X-45 (A) and 60 μM Triton X-100 (B). The neurons were exposed to the detergents for about 60 min.

remained nearly unchanged for more negative potentials. A possible cause for the increased slope conductance and the concomitant decrease in potential at zero current after prolonged exposure to the detergent might be a deterioration of the neuron due to the penetration of two microelectrodes and the repetitive extensions. It was usually not possible to reverse the effect of the detergent in the voltage clamp situation in contrast to measurements with only one micropipette (cf Fig 3) or with shorter time of exposure.

Membrane currents induced by stretch. The records in Fig. 5 show examples of the stretch induced current (SIC) during voltage clamp at various membrane potentials before and after application of Triton X-45. The current induced at resting potential was inward and had a configuration and time course closely similar to the potential change induced by the same stretch. The SIC decreased at positive polarization values and disappeared at a membrane potential of 15 mV At more positive membrane potential values the SIC due to extensions reversed in direction. The time course of the SIC was otherwise unaffected by the membrane potential.

After about 60 min in Triton X-45 there was a marked decrease of the SIC for potential values less than the reversal potential (which was about 20 mV) whereas the SIC appeared to be unchanged or somewhat enhanced for potential values more positive than the reversal potential. After 90 min in Triton X-45 the SIC had diminished still more while the reversal potential remained unchanged (+17 mV).

Fig. 6 shows the I–V relationship of the passive membrane current (solid symbols) and the SIC (open symbols) at different membrane potentials during voltage clamp before and after application of Triton X-45 and Triton X 100. The SIC obtained at each value of E_m is represented by open circles in normal *Astacus* saline.

A comparison between the curves for Triton X-45 and Triton X-100 shows that the effect of Triton X 100 on the SIC was almost identical t that produced by Triton X-45 The change in the reversal potential varied from one cell to another but was always in the nega-

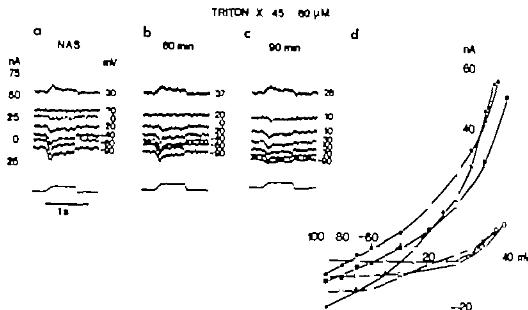


Fig. 5 Receptor membrane currents during rest and stretching in normal saline and after exposure of cell to detergent. Triton X-45. *a–c* Records of SIC (stretch induced current) at different membrane potential levels in normal *Astacus* saline (*a*), after 60 min (*b*) and 90 min (*c*) in 60 μM Triton X-45. Stretch was applied after the membrane current had reached a steady level. Holding potentials for each recording are shown to the right. *d* I V relations of the steady-state passive current (solid symbols) and SIC (open symbols). SIC was obtained by subtracting the steady-state passive current from the peak membrane current during stretch. Circles normal *Astacus* saline squares after 60 min in Triton X-45 triangles, after 90 min in Triton X-45

lowed after about 10–15 min by the decrease of the static phase. Complete abolition of the response did not occur until after a total exposure time of about 90 min the dynamic phase of the response being the last to disappear

With return to normal solution the dynamic phase of the receptor potential first returned followed later by the dynamic impulse response and still later by the impulses during static extension until the response was almost completely restored. The effect of Triton X 100 on the receptor potential in all respect resembled that of Triton X-45 as can be seen in Fig. 3 *B*.

In summary the effects of Triton X-45 and Triton X 100 consists of an initial blocking of the impulse generating mechanisms of the neuron followed by a later blocking of the transducer action These effects were produced with small changes of the resting membrane potential

The effect of detergents examined with voltage clamp

Membrane currents in the resting neuron As can be seen in Fig. 5 and Fig. 6 Triton X-45 and Triton X 100 have little effect on the passive properties (filled symbols) of the receptor neuron The cell illustrated in Fig. 5 was exposed to Triton X-45 (60 μM) for about 90 min. In normal *Astacus* saline (NAS) this cell had a membrane resistance of about 3 7 MΩ at resting membrane potential this value did not change during exposure to the detergent for 60 min although the cell was polarized to about −40 mV After 90 min the slope conductance had increased for potentials more positive than the resting membrane potential while it

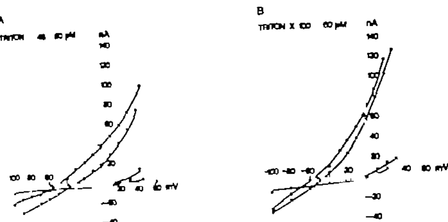


Fig. 6. I–V relations of the steady-state passive currents (solid symbols) and SIC (open symbols) measured as in Fig. 5 before and after treatment of the cell with 40 μM Triton X-45 (A) and 60 μM Triton X 100 (B). The neurons were exposed to the detergents for about 60 min.

remained nearly unchanged for more negative potentials. A possible cause for the increased slope conductance and the concomitant decrease in potential at zero current after prolonged exposure to the detergent might be a deterioration of the neuron due to the penetration of two microelectrodes and the repetitive extensions. It was usually not possible to reverse the effect of the detergent in the voltage clamp situation in contrast to measurements with only one micropipette (*cf.* Fig. 3) or with shorter time of exposure.

Membrane currents induced by stretch. The records in Fig. 5 show examples of the stretch induced current (SIC) during voltage clamp at various membrane potentials before and after application of Triton X-45. The current induced at resting potential was inward and had a configuration and time course closely similar to the potential change induced by the same stretch. The SIC decreased at positive polarization values and disappeared at a membrane potential of 15 mV. At more positive membrane potential values the SIC due to extensions reversed in direction. The time course of the SIC was otherwise unaffected by the membrane potential.

After about 60 min in Triton X-45 there was a marked decrease of the SIC for potential values less than the reversal potential (which was about +20 mV) whereas the SIC appeared to be unchanged or somewhat enhanced for potential values more positive than the reversal potential. After 90 min in Triton X-45 the SIC had diminished still more while the reversal potential remained unchanged (~17 mV).

Fig. 6 shows the I–V relationship of the passive membrane current (solid symbols) and the SIC (open symbols) at different membrane potentials during voltage clamp before and after application of Triton X-45 and Triton X 100. The SIC obtained at each value of E_m is represented by open circles in normal *Astacus* saline.

A comparison between the curves for Triton X-45 and Triton X 100 shows that the effect of Triton X 100 on the SIC was almost identical to that produced by Triton X-45. The change in the reversal potential varied from one cell to another but was always in the nega-

tive direction for both detergents (towards more negative potential values). In an earlier investigation on the stretch receptor (Brown, Ottoson and Rydqvist, to be published) it was observed that the reversal potential appeared to vary with the length and configuration of the main dendrites and that the reversal potential generally was more positive for neurons with a long main dendrite than for neurons with short richly branching dendrites. It is possible therefore that the differences in reversal potential observed might partly be attributed to structural differences of the dendritic tree of the neurons.

Discussion

The present results show that both Triton X-45 and Triton X 100 block the action potential and the receptor potential in the crustacean stretch receptor neuron. At a concentration of 60 μM the conducted impulses are blocked within 20–30 min and this effect is produced without any appreciable change of the resting membrane potential. It is most likely that the effect on the conducted activity can be ascribed to a block of the potential dependant sodium permeability of the impulse generating membrane as judged from the measurements of the maximum rate of rise of the action potentials. As shown by Hodgkin and Katz (1949) and by Schwartz, Ulbricht and Wagner (1973) this parameter is a good approximation of the sodium current (*cf.* Rydqvist 1977).

The voltage clamp experiments show that the passive membrane properties *i.e.* the slope conductance was very little affected by the two detergents. In some experiments there was a clear decrease in the slope conductance for potential values more positive than the resting membrane potential. The same effect was seen in preparations soaked in normal *Astacus* saline and appears therefore not to be caused by the detergents. It was also noted in many experiments that the resting membrane potential was reduced after a series of clamping measurements. Following recovery at the resting membrane potential (without being in the clamp-condition) the resting membrane potential usually returned to the pre-clamp values.

As shown, Triton detergents cause large reduction of the inward (negative) stretch induced current (SIC). A tentative explanation to this effect is that the detergents have a selective action on the sodium permeability of the receptor membrane. It is probable that the sodium equilibrium potential of the neuron is 40 to 60 mV. A selective decrease of the sodium permeability would therefore diminish the SIC for negative potential values and consequently also the receptor potential. A decrease in sodium permeability of the receptor membrane could be due to a lyotropic interaction between the detergents and the lipid components of the membrane. It is well known that detergents can interact with the lipid bilayer of biological membranes, changing it from the naturally occurring liquid crystal lamellar phase to either a less or more fluid liquid crystalline state or towards the so called gel state (Chapman 1971, Small *et al.* 1966, Small 1968, Wojtowicz 1975). The change to a more stable structure might explain the decrease in sodium permeability. Another possibility is that the detergent reacts with specific sites in the membrane (*e.g.* a protein). In view of the fact that the receptor potential was not affected until after prolonged exposure of the neuron to the detergents this alternative appears less probable at least if it is assumed

that the sites are located at the outer side of the membrane. The slow action on the receptor membrane might indicate that there is an outside diffusion barrier or that the site of action is intracellular.

It is in this context of interest to note that it has been observed in experiments on the motor end plate in frog skeletal muscles that Triton X-100 causes disruption of the membrane of mitochondria (Rydqvist, unpublished observations). It is therefore possible that the effects observed in the present experiments may partly be attributed to an intracellular release of calcium from mitochondria.

The potential at which the SIC reverses direction is invariably changed towards more negative values after treatment with Triton detergents. No appreciable difference between the two detergents was observed. Changes in reversal potential must be due to change in electrolyte composition either on the outside or on the inside of the cell membrane. The composition of the outside was maintained constant and consequently the most likely change is a decrease in intracellular potassium with a concomitant increase in intracellular sodium. As pointed out earlier the permeability of the membrane for sodium is probably reduced by the detergents and therefore it seems unlikely that the change in reversal potential is caused by an increase of intracellular sodium $[Na]_i$ due to an increase in the net sodium flux. A tentative explanation may be that the pumping mechanism in the membrane is inhibited and therefore the extrusion of sodium diminished. As a consequence of this the interior of the cell would gain sodium and loose potassium thereby changing the reversal potential for the SIC.

In summary it thus would appear that Triton detergents mainly affect the properties of the active membrane of the stretch receptor neuron. The impulse generation is considerably more sensitive to the action of Triton detergents than the transducer action while the spikes are blocked within 20–30 min the transducer functions of the neurons are not abolished until after 90 min. These effects are produced without significant changes of the resting membrane potential. The conclusion therefore is that Triton X-45 and Triton X 100 block not only the sodium channels involved in the generation of impulses but also the sodium current of the transducer membrane. The mechanism for this selectivity is unknown at present. The permeability for other ions involved in the production of the conducted and the non-conducted activity of the cell may also be affected.

The authors are greatly indebted to Prof. H. M. Brown for valuable discussions during the preparation of the manuscript. We wish also to thank Mrs Marit Hahn for technical assistance.

This work has been supported by grants from Karolinska Institutet and from the Swedish Medical Research Council, project no. B77-14X-00043-13B.

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Acta physiol. scand. 1978. 103. 19–30

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Capillary supply of the muscle fibre population in hindlimb muscles of the cat

By

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Received 28 October 1977

Abstract

MYRHAGE, R. *Capillary supply of the muscle fibre population in hindlimb muscles of the cat.* Acta physiol. scand. 1978. 103. 19–30.

Comparative analyses of the fibre content (FG, FOG, and SO fibres) and the capillary density (the number of capillaries surrounding individual fibres and the capillary/fibre ratio) were performed in hind limb muscles of the cat. Cross-sections from the tenuissimus, the biceps femoris, the lateral head (LG) and the medial head (MG) of the gastrocnemius and the soleus were cut in a cryostat. The sections were stained histochemically for the NADH$_2$-diaphorase and alkaline (pH 9.4) actomyosin ATPase activity which enables differentiation of different types of fibres. The endothelium of the capillaries was identified on staining for unspecific alkaline ATPase activity. The number of capillaries surrounding each individual muscle fibre had a positive correlation, first to the oxidative capacity and secondly to the average diameter of the fibres. The thin tenuissimus muscle did not differ in this respect from the thicker muscles. The highest proportion of SO fibres was found in the soleus and the MG muscles. FG fibres of two different types were dominating the fibre mass in the biceps femoris and the LG muscles, while the tenuissimus contained more FOG fibres than these muscles. In general the FG fibres had a larger diameter than the FOG and the SO fibres. The soleus and the MG muscles contained larger fibres than the other examined muscles. FG fibres were surrounded by fewer capillaries than FOG and SO fibres. The soleus and the MG muscles, with higher percentage of SO fibres and also larger fibres, had the largest number of capillaries around the fibres and the highest capillary/fibre ratio.

Key words: Hind limb muscles, muscle fibre population, muscle fibre diameter, capillary density

The microvascular bed in the cat tenuissimus muscle has previously been analysed with intravital microscopy (Eriksson and Myrhage 1972). Since this muscle is rather thin and small it is not unequivocally representing skeletal muscle tissue in wider aspects. The present study deals with comparative analyses of the muscle fibre population and the capillary supply in the tenuissimus and thicker hind limb muscles in cats.

Detailed knowledge of the fibre composition in various skeletal muscles has been provided by histochemical techniques (see review by Close 1972, Khan 1976). Quantitative biochemical analyses have also been performed on muscles with known fibre content (Dawson and Romanul 1964, Peter *et al.* 1972).

Romanul (1965) found that the number of capillaries, surrounding each individual muscle fibre, was directly proportionate to the activity of oxidative enzymes in the fibres. Similar observations have been reported by *Andersen (1975). Contradictory to this,* Plyley and Groom (1975) stated that the *diameter* of the muscle fibre is the only factor determining the number of capillaries in muscle tissue. However, this statement was not based on actual measurements of the muscle fibre diameter.

Ariano *et al.* (1973) have described the percentage distribution of different muscle fibre types in various cat muscles. There is, however, no comparative study available concerning the capillary density in these muscles, even if quite a few authors give data on capillary density in muscles of different fibre composition (see *e.g.* Hudlická 1973).

The present study will discuss the relationship between fibre type, fibre dimension and capillarization of different fibres in some cat hind limb muscles. The different muscle fibre types will be described as FG (fast twitch – glycolytic), FOG (fast twitch – oxidative-glycolytic) and SO (*slow twitch – oxidative). These terms were proposed by Peter et al.* (1972) and are corresponding to the FF, FR, and S fibres defined by Burke *et al.* (1971). FG, FOG and SO are also corresponding to the terms A, B and C fibres, respectively, in previous publications (Eriksson and Myrhage 1972, Myrhage and Hudlická 1976).

Materials and Methods

8 adult cats of both sexes, weighing about 2.5 kg, were used. They were anesthetized with chloralose (i.v. injection of 50 mg/kg) after induction with ether.

The examined muscles (the tenuissimus, the dorsal part of the biceps femoris, both heads of the gastrocnemius, and the soleus) were carefully exposed and transverse segments, 10 mm long, were excised from 3 different parts of each muscle (15 mm distal to the origin and 15 mm proximal to the insertion and one segment from the centre of the muscle belly). In the tenuissimus muscle, however, one segment was taken from the usual area of intravital microscopy, which is located about 10 mm distal to the nutritive artery emerging from the popliteal fat pad (*cf.* Myrhage and Eriksson 1977). The other two segments were taken 20 mm proximal respectively distal to this area.

The muscle segments were kept in *icecold Histocon (Histo-Lab, Göteborg, Sweden) for up to 1 h,* thereafter freezing was performed in liquid propane-propene (Gasol). After mounting on cryostat chucks the specimens were stored at −70°C. In a cryostat (−20°C), serial sections (7–10 μm thick) were cut perpendicularly to the muscle fibre direction. To enable qualitative fibre differentiation, the sections were stained histochemically for the activity of NADH$_2$-diaphorase (showing the oxidative capacity (Chayen *et al.* 1973)) and alkaline (pH 9.4) actomyosin ATPase (Padykula and Herman 1955). The method of unspecific alkaline ATPase described by *Guth and Samaha (1969, 1970)* was used for histochemical staining of capillary endothelium.

In 3 of the 8 animals the hind legs were fixed by 3% glutaraldehyde (buffered with 0.075 M sodium cacodylate to pH 7.2) via perfusion, at a pressure of 13.3 kPa (100 mmHg), through a cannula placed in abdominal aorta. To optimize the filling of the microvascular bed, vasodilation was induced with papaverine (0.– *mg/kg*) and preperfusion was undertaken with Perfadex[R] (*cf.* Myrhage and Eriksson 1977). Muscle segments, as described above, were excised and embedded in Epon and serial sections (1 μm thick) were cut, perpendicular to the muscle fibres, in an ultrotome. In sections, stained with toluidine blue, the capillaries could easily be identified (Eriksson and Myrhage 197).

For description *of the fibre population* in the examined muscles, 10 entire muscle fibre bundles (*i.e.* 300–400 fibres) were evaluated in each of the serial sectioned muscle segments. From each section a train of bundles, extending from the central area towards the periphery of the muscle belly, was photographed in a Leitz Orthomat automatic microscope camera (Leitz-Wetzlar, W. Germany). By comparing photographs from serial sections, alternately stained for diaphorase-, actomyosin ATPase- and unspecific alkaline ATPase activity, different fibre types could be distinguished and quantified. Fibre diameters were calculated

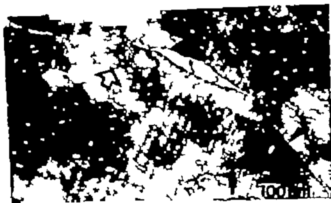


Fig. 1 Ultrathin section from the medial head of the gastrocnemius muscle (glutaraldehyde fixation). Filled arrows indicate the boundary of muscle fibre "bundle", open arrows show the capillaries.

by taking the mean of 4 polygonal diameter measurements, on each of the studied fibres, in calibrated photographs (Cotter et al. 1973). The number of capillaries surrounding each muscle fibre was simultaneously registered.

Both glutaraldehyde fixed sections and sections stained for unspecific alkaline ATPase activity were used for evaluation of capillary density. The numbers of capillaries per muscle fibre (the capillary/fibre ratio) and per cross sectional area were calculated in 20 adjacent fibre bundles from each muscle segment.

Comments on methods

The muscle fibre bundle

Because of its fairly uniform fibre arrangement, the bundle (Fig. 1 and 2) was judged to be the most representative unit for descriptions of the fibre population. The bundle is enclosed by a delicate sheath of perimysium and contains about 20–80 muscle fibres, which are surrounded by a thin layer of endomysium (Myrhage and Eriksson 1977). The entire fibre mass was grouped into fibre bundles in all the examined muscles. In the tenuissimus, biceps femoris and gastrocnemius muscles most of the large fibres, with low oxidative capacity were located close to the periphery of the bundles, while the smaller fibres with higher oxidative capacity were situated deeper in the bundles.

Capillary visualization

In muscle sections stained ad modum Padykula and Herman (1955), the alkaline ATPase activity in the different muscle fibres is marked quite intensively. The capillaries are, however, only weakly and unevenly visualized. In the sections stained for unspecific alkaline ATPase activity (Guth and Samaha 1970) the capillaries are distinctly delineated (Fig. 2d). Capillary endothelium has been shown to contain a high activity of alkaline phosphatase (Gomori 1939). ATP (which is used as substrate in methods for ATPase) can be hydrolysed by alkaline phosphatase as well as by different specific ATPases (Padykula and Herman 1955, Freeman and Kaplan 1960). Since the method by Guth and Samaha (1970) contains no specific inhibitor for alkaline phosphatase, it seems to be quite comparable with the method of Gomori (1939) and all capillaries, present in the section, should be stained by either of these two methods.

The capillaries can also be identified in muscle sections fixed by glutaraldehyde (Fig. 1; see also Eriksson and Myrhage 1972).

Results

Muscle fibre orientation

The muscle fibres in the tenuissimus muscle run parallel to the longitudinal axis of the muscle. A similar relationship existed in the dorsal part of the biceps femoris and in the

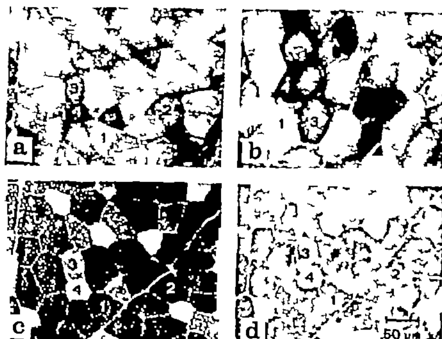


Fig. 2. Cryostat sections from the lateral head (a, c and d) and the medial head (b) of the gastrocnemius muscle. Histochemical staining for $NADH_2$-diaphorase activity (a, b), alkaline ATPase activity *ad modum* Padykula and Herman (c) and *ad modum* Guth and Samaha (d). Fibre types: FG (1), FG/FOG (2), FOG (3) and SO (4).

lateral head of the gastrocnemius (LG). In the medial head of the gastrocnemius (MG) and in the soleus muscle the fibres were, however, oriented obliquely to the longitudinal axis of the muscle (Myrhage and Eriksson 1977).

Fibre populations

Different fibre types could be distinguished by their staining intensity for alkaline ATPase and $NADH_2$-diaphorase-activity (denoted here as ATPase activity/diaphorase activity): FG – high/low, FOG – moderate/high close to the sarcolemma but low to moderate in the centre of the fibre, FG/FOG – high/as in FOG fibres, SO – low/moderate to high, but evenly distributed (Fig. 2).

FG and FOG fibres, taken together, were most common in the tenuissimus, the biceps femoris and the LG muscles, where such fibres constituted about 85 % of the total fibre population. More than half of this amount consisted of FOG fibres in all examined tenuis-

Table I. Muscle fibre populations (mean ± S.E. –4).

Fibre types (%)	Tenuissimus	Biceps femoris	Gastrocnemius		Soleus
			LG	MG	
FG	35±1.1	41±2.3	50±0.8	4[illegible]±1.1	—
FG/FOG	—	12±1.1	11±0.9	—	—
FOG	49±2.1	33±2.1	24±0.9	22±1.8	1±0.4
SO	16±1.1	14±2.0	15±0.6	36±1.3	99±0.4

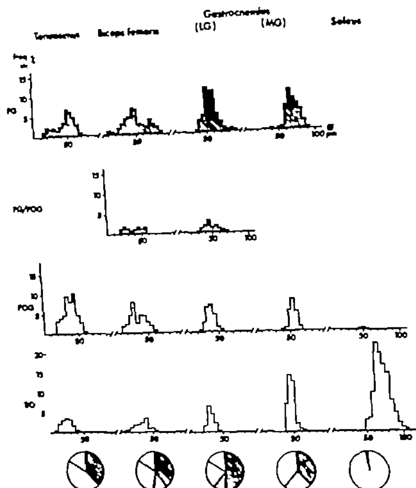


Fig. 3. Frequency distribution of fibres of different type and diameter (percentage values from one animal). The total percentage of each fibre type is illustrated as sectors at the circles.

semus muscles, while the FG fibres were dominating in the biceps femoris and the LG muscles. FG/FOG fibres were found, as a regular fibre type only in the LG and the biceps muscles, where they formed 11 to 12% of the total fibre population (Table I). The highest content of SO fibres was found in the soleus muscles (on average 99 ±0.4%/mean ± S.E., 4) and in the MG muscle (on average 36 ± 1.3%).

Fibre dimensions

The FG fibres had the largest average diameter of the fibre population, in all evaluated segments from the tenuissimus, biceps, LG and MG muscles (Fig. 3 and Table II).

The MG muscles contained the largest FG fibres (average diameter 69 ± 0.7 µm) in all the animals. These fibres were considerably larger than in the LG muscles (on an average:

TABLE II Average diameters of muscle fibres (μm mean ± S.E.; n = 4).

Fibre types	Tenuissimus	Biceps femoris	Gastrocnemius		Soleus
			LG	MG	
FG	47±2.4	50±2.6	57±1 8	69±0.7	—
FG/FOG	—	37±2.5	48±1 7	—	—
FOG	38±1.8	35±1 7	39± .0	55±0.6	51±1.0
SO	.5±1 7	3 ±2.9	33±1 4	47±0 4	72±1 9

57 ± 1 8 μm) In the tenuissimus and the biceps muscles, the dimensions of the FG fibres were smaller and not different from each other (average values 47 ± 2.4 μm and 50 ± 2 6 μm, respectively)

The range of the FG fibre diameters was widest in the biceps femoris and the tenuissimus muscles, in all animals, as shown in Fig. 3

The FOG fibres had a similar average diameter in the tenuissimus, biceps and the LG muscles (average values 38 ± 1 8, 35 ± 1 7 and 39 ± 2.0 μm, respectively). In the MG muscles they were, however considerably larger (55 ± 0 6 μm) In the soleus muscles there were only quite few FOG fibres (always less than 3 % of the evaluated fibre mass) and these fibres had an average diameter comparable to the same type of fibre in the MG muscles (Table II)

The transitional group of fibres (FG/FOG) with a high activity of alkaline ATPase but with an oxidative capacity similar to the FOG fibres, also had an average diameter similar to FOG fibres in the biceps muscles. In the LG muscles the diameters of the FG/FOG fibres were ranging inbetween the values of FG and FOG fibres (see Table II).

The SO fibres were considerably larger in the MG muscles (47 ± 0.4 μm) as compared to the tenuissimus, biceps and LG muscles (25 ± 1 7 32 ± 2.9 and 33 ± 1 4 μm, respectively). In the tenuissimus and the biceps muscles quite small SO fibres (about 10–15 μm in average diameter) were found to a varying extent in all segments from these muscles, as shown in Fig. 3

The soleus muscles were almost entirely (97–100 %) composed of SO fibres with an average diameter of 72 ± 1 9 μm.

Number of capillaries around fibres

The absolute values for the number of capillaries around different muscle fibres varied from 0–5 in the tenuissimus, biceps and the LG muscles, from 0–7 in the MG muscles and from 2–9 in the soleus muscles.

In spite of its larger fibre diameter the FG fibres, in all examined tenuissimus, biceps, LG and MG muscles, had a smaller number of surrounding capillaries (range 1 5–3 3) than the FOG fibres (range 2.0–3 8). The few FOG fibres found in the soleus muscles, had an even higher number of surrounding capillaries (on an average 4.8). For the FG/FOG fibres the number of fibre surrounding capillaries were in the range of the figures for the FG and the FOG fibres (Fig. 4 and 5).



Fig. 4. Capillary density in cryostat sections from the tenuissimus ( ), the dorsal part of the biceps femoris (b), the lateral head (c) and the medial head (d) of the gastrocnemius and the soleus (e). All these sections are stained for unspecific alkaline ATPase ad modum Gath and Samaha. The even distribution of formazan granules in section from the soleus (stained for $NADH_2$-diaphorase activity) is shown in (f). Fibre types. (1) FG (2) FG/FOG (3) FOG (4) SO

The SO fibres in the soleus muscles had a similar number of surrounding capillaries as the FOG fibres (average values 5.0 and 4.8, respectively). In the other 4 muscles the SO fibres had number of surrounding capillaries (range: 2.0–3.4) which was slightly lower or similar to the corresponding values for the FOG fibres (Table III).

As summarized in Fig. 5 a, the biceps femoris and the LG muscles had fewer capillaries around their fibres as compared to the MG and the soleus muscles, where also all types of fibres had larger average diameters. Furthermore, the FG (and FG/FOG) fibres were dominating in the biceps and the LG muscles, while the MG and the soleus muscles had more of SO fibres. The tenuissimus muscles had a higher content of FOG fibres and these muscles fell inbetween the biceps-LG and the MG-soleus groups regarding the number of fibre surrounding capillaries.

The fibre diameter and the number of surrounding capillaries in different muscles had a positive correlation (Fig. 5 b), however the FG and the FG/FOG fibres had notably lower values for the number of capillaries around fibres.

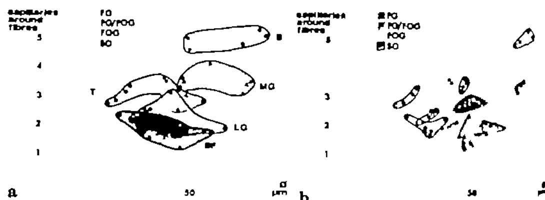


Fig. 5 The average number of capillaries around individual muscle fibres, in relation to fibre type and average diameter of the fibres (in four animals). a) values grouped for different muscles, b) values grouped for different fibre types. Te = tenuissimus BF = biceps femoris, LG = the lateral head and MG = the medial head of the gastrocnemius S = the soleus.

Capillary density

The capillary/muscle fibre ratio was calculated from examinations of 20 adjacent muscle fibre bundles in each muscle segment and the average values for the animals ranged from 0 78–1 92 in sections stained for unspecific alkaline ATPase and from 0 76–1.87 in the sections from glutaraldehyde perfused muscles.

The biceps femoris and LG muscles had the lowest values (range in 7 animals 0.74-0.89) and the MG and soleus muscles had the highest values (range 1 04–1 17 and 1.83–1.94, respectively). The tenuissimus muscles showed intermediate values, ranging from 0.89 to 1 02.

The capillary density per mm of cross section area was 657 ± 54 (mean ± S E., n = 4) for the tenuissimus muscle. The corresponding values for the biceps femoris, the LG the MG and the soleus muscles were 617 ± 66 570 ± 34 695 ± 49 and 948 ± 47 respectively

In general the values from the glutaraldehyde perfused muscles were only slightly smaller than the corresponding values from the muscles stained for activity of unspecific alkaline ATPase (Table III)

TABLE III Average number of capillaries around muscle fibres and capillary/fibre ratio (mean ± S E)

Fibre types	Tenuissimus	Biceps femoris	Gastrocnemius LG	Gastrocnemius MG	Soleus
Capillaries around muscle fibres( )					
FG	1.7 ± 0.11	1.5 ± 0.11	1 9 ± 0.09	3.3 ± 0.09	—
FG/FOG	—	1.8 ± 0.13	1.9 ± 0.15	—	—
FOG	1 4 ± 0.09	2.0 ± 0.06	2.8 ± 0.13	3.8 ± 0 13	4.8 ± 0 10
SO	1.9 ± 0.09	2.0 ± 0 14	2.3 ± 0 09	3.4 ± 0 09	5.0 ± 0 11
Capillary/fibre ratio					
(*)	0.95 ± 0.03	0.78 ± 0 09	0.83 ± 0.08	1 13 ± 0.02	1 92 ± 0 01
( )	0 92 ± 0.02	0.79 ± 0.02	0.76 ± 0.01	1 10 ± 0.04	1.87 ± 0.02

( ) from sections stained for ATPase (n = 4).
( ) from glutaraldehyde perfused muscles (= 3).

Discussion

The present results indicate that the amount of capillaries in skeletal muscle tissue has a positive correlation firstly to the oxidative capacity of different muscle fibres, and secondly the dimension of the fibres.

Muscle fibre populations

The medial head of the gastrocnemius (MG) was found to contain more slow twitch-oxidative (SO) fibres than the lateral head (LG) of the same muscle. The LG muscle had essentially the same fibre population as the biceps femoris muscle. This is in accordance with the findings by Ariano *et al.* (1973).

The content of fast twitch-oxidative-glycolytic (FOG) fibres in the tenuissimus (and also in the biceps, LG and MG muscles) was higher in the present study as compared to figures presented by Ariano. The discrepancy might be due to a higher level of exercise in the cats used in the present study since 75% of them had been confined to cages for only 3–4 weeks before the expts. It has been shown that exercise increases the activity of oxidative enzymes in muscle (*cf* Holloszy *et al.* 1971) and also the number of muscle fibres corresponding to the FOG type (Barnard *et al.* 1970, Jansson and Kaijer 1977).

Almost all fibres in the soleus muscles from the present expts. had an even distribution of formazan granules ($NADH_2$-diaphorase reactions) which is typical for SO fibres. This finding corresponds well with observations by several authors (*e.g.* Tsukamoto and Mori 1966, Ariano *et al.* 1973 Hammarberg 1974).

A fraction of fast twitch-glycolytic (FG) fibres, with a notably high activity of $NADH_2$-diaphorase (similar to the one found in FOG fibres) was found in the biceps and the LG muscles. This type of fibre was also observed by Hammarberg (1974) in the cat LG muscle.

Muscle fibre diameters

The degree of muscle tension would not affect the fibres diameter values, since fibre perpendicular segments of the examined muscles were frozen instead of whole muscles.

The FG fibre was the largest fibre type in the tenuissimus, biceps femoris, LG and MG muscles. The average diameter of the FOG and SO fibres was about 20–25% smaller than that of the FG fibres, and the SO fibres were generally slightly smaller than the FOG fibres. All 3 fibre types in the MG muscles were considerably larger than corresponding fibres in the tenuissimus, biceps and LG muscles. The soleus muscle contained SO fibres with an average diameter about 50% larger than the SO fibres in the MG muscles and more than 100 larger as compared to the corresponding fibres in the three other muscles.

The fibre dimensions in the tenuissimus and the MG muscles are quite similar to the data presented by Boyd (1956) and Henneman and Olson (1965). In the present study the 4 different fibre types of the LG muscle are all smaller than the corresponding figures reported by Hammarberg (1974). The method for estimation of fibre diameters used by Hammarberg was "the mean of the two largest diameters" while the present estimations were based on 4 polygonal diameters. This would reasonably explain the difference in diameter values in

these two studies. Furthermore, entire, adjacent muscle fibre bundles were examined in the present study instead of "randomly chosen areas" as in the study by Hammarberg.

The average fibre diameter for the soleus muscles, in the present study is similar to the values found by Hammarberg, but about 15% larger than the figure presented by Henneman and Olson (1965).

The frequency distribution for the fibre diameters had a wide range in the tenuissimus, biceps and LG muscles, which all have fibres oriented parallel to the longitudinal axis of the muscle belly. Especially the tenuissimus and the biceps muscles contained several quite small fibres (10–15 μm in diameter). For the tenuissimus muscle, this could be explained by the existence of interdigitating, only 2 cm long muscle fibres as described by Adrian (1925).

Capillary supply of muscle fibres

The larger fibres in the MG and soleus muscles were surrounded by more capillaries than the fibres in the biceps femoris and LG muscles. In general, the FOG and the SO fibres had slightly higher values of surrounding capillaries than the FG and the FG/FOG fibres.

Since the diameters of the fibres in the tenuissimus were *not* exceeding the diameters of fibres in the biceps and LG, the numbers of fibre surrounding capillaries in the tenuissimus muscle were comparably large. This might be explained by the high proportion of FOG fibres found in the tenuissimus muscle. Several FG fibres would accordingly share their capillaries with bordering FOG fibres.

The high number of fibre surrounding capillaries found in the soleus and the MG muscles as well as the lower values for the biceps and LG and the medium values for the tenuissimus were confirmed by the calculated figures of capillary/muscle fibre ratio. The sections stained for unspecific alkaline ATPase and the sections from the glutaraldehyde perfused muscles had approximately the same capillary density.

The number of capillaries around each fibre increased parallel with increasing muscle fibre diameter. However, the FOG and SO fibres were surrounded by more capillaries than the comparatively larger FG fibres, which means that muscle fibres with high oxidative metabolism were found to be supplied by more capillaries than fibres with low oxidative metabolism.

This is in accordance with findings presented by *e.g.* Ranvier (1874), Stoel (1925), Lee (1958) and Romanul (1965). Similar findings were also reported by Andersen (1975), while Plyley and Groom (1975) stated that, the muscle fibre diameter was the only factor determinating the number of capillaries surrounding the fibre.

A direct relationship between oxidative capacity of muscle fibres and the capillary density in the muscle has also been indicated by physiological experiments. Hilton (1968, Hilton *et al.* 1970) observed that a "red" muscle, with high oxygen consumption (cat soleus) had a maximal blood flow capacity, which was 2–3 times higher than that of the "white" gastrocnemius muscle. This finding was confirmed by Folkow and Halicka (1968) and by Reis and Wooten (1970).

Furthermore, chronic stimulation of rabbit fast muscles at a low impulse frequency (10 Hz) has been shown to increase the activity of oxidative enzymes but decrease the average diameter of the muscle fibres. Still the capillary/fibre ratio increased by 20% after only

days and by 55% after 28 days of stimulation (Cotter *et al.* 1973, Pette *et al.* 1973, Brown *et al.* 1976, *cf.* Myrhage and Hudlická, 1977).

Thus, the activity of oxidative enzymes in the individual muscle fibres seems to be, even more important than the fibre diameter for the capillary density and reasonably also for the number of capillaries surrounding each fibre in skeletal muscle tissue. The present study shows that the tenuissimus muscle in cat is not different from thicker hindlimb muscles in this respect.

This study has been supported by grants from the Faculty of Medicine, University of Göteborg, the Göteborg Medical Society, Wilhelm and Martina Lundgrens Vetenskapsfond, Svenska Sällskapet för Medicinsk Forskning, Emma Johanna Jonssons Donationsfond, University of Göteborg.

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Acta physiol. scand. 1978. 103. 31–39

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Histochemical and biochemical changes in human skeletal muscle with age in sedentary males, age 22–65 years

By

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Received 31 October 1977

Abstract

LARSSON, L., B. SJÖDIN and J. KARLSSON. *Histochemical and biochemical changes in human skeletal muscle with age in sedentary males, age 22–65 years.* Acta physiol. scand. 1978. *103*. 31–39

Biopsies for histochemical and biochemical analyses were taken from the vastus lateralis muscle of 55 untrained healthy male subjects from 22 to 65 years of age. Fibre type distribution changed towards a decrease in the percentage of type II fibres, both in type IIA and type IIB fibres, whereas type IIB/IIA fibre ratio and type IIC percentage did not change with increasing age. It was found that the type IIB/IIA fibre ratio was inversely related to type I fibres, *i.e.* subjects rich in type I fibres had a relatively smaller proportion of type IIB fibres. Fibre area determinations revealed a selective decrease in type II fibre area. Consequently the type II/I fibre area ratio and relative type II fibre area decreased. No changes in the specific activities of Mg^{2+} stimulated ATPase and myokinase were observed, while the activity of lactate dehydrogenase (LDH) was higher in the youngest groups than in the oldest. LDH isozyme pattern shifted towards a decrease in percentage distribution of the muscle specific isozymes and a corresponding decrease in muscle specific activity while the activity of the heart specific isozymes did not change.

During the main part of man's productive period of life, ageing processes take place to varying degrees in all organ systems. Perhaps some of the most prominent changes are the decrease in muscular strength and decline in the *fine* co-ordination of movements (Ufland 1933, Birren 1952, Burke *et al.* 1953, Asmussen and Heebøll-Nielsen 1961). It is reasonable to assume that one important part of the ageing process causing these deficiencies takes place in muscle tissue itself. The objective of the present study has been to investigate the histochemical and some of the biochemical characteristics of importance for muscle contractility and endurance of a relatively homogeneous male population in terms of occupational and spare time physical activity in the age range of 22 to 65 years.

Material and methods

55 healthy male volunteers (22–65 yrs) participated in the study. They were divided into different age groups, 20–29, 30–39, 40–49, 50–59 and 60–65 years. The number of subjects per age group varied from

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TABLE I Fibre type distributions, fibre areas, fibre area ratios and relative fibre area of type II fibres in the muscle of the different age groups studied.

Age group	Mean age yrs	Number	% Type I fibres	Area μm^2 Type I	Area μm^2 Type II	Area ratio Type II/I	% Area Type II fibres
20–29	26	11	40.5±3.9 (14–59)	5 666±499 (2 851–8 704)	6 933±388 (4 562–9 505)	1.24±0.05 (1.04–1.60)	63.7±4.3 (43.1–89.2)
30–39	36	12	36.5±1.6 (27–44)	6 344±597 (3 963–8 931)	6 975±316 (4 872–8 981)	1.17±0.13 (0.69–2.05)	67.0±2.1 (55.5–72.9)
40–49	43	10	48.1±4.2 (29–67)	6 734±398 (4 939–8 670)	6 627±340 (4 224–9 558)	0.98±0.06 (0.84–1.29)	50.9±3.8 (31.8–63.9)
50–59	53	12	51.7±3.0 (28–68)	5 941±627 (2 663–9 005)	5 954±449 (4 072–8 003)	1.04±0.09 (0.61 1.69)	49.3±4.5 (37.2 72.0)
60–65	6.	10	55.0±4.5 (38–79)	5 591±343 (4 120–7 275)	5 241±203 (4 230–6 437)	0.96±0.05 (0.74–1.18)	43.6±4.3 (18.8–65.4)

Values are means ± S.E. Values in parentheses are highest and lowest observations.

fibre area ratio decreased (p 0.07) with age (Fig. 2). Consequently the relative type II fibre area decreased (p 0.001) from 64% to 44% in the 20–29 and 60–65 year old groups, respectively.

No significant change in the enzyme activity of Mg stimulated ATPase or myokinase (MK) with age was observed. However lactate dehydrogenase (LDH) activity was found to be significantly (p 0.05) higher in the younger groups as compared with the older. LDH isozyme pattern shifted towards less muscle-specific isozymes with age as indicated by the percentage distribution M LDH (p 0.01) (Table III). This took place by means of a significant (p 0.01) decrease in the activity of muscle-specific isozymes with age. The activity of heart-specific isozymes tended to increase although not significantly (Fig. 3). Consequently the M/H activity ratio decreased (p 0.02).

TABLE II Fibre type distribution as type I, IIA, IIB and IIC in 41 of the 55 subjects in Table I.

Group	Mean age yrs	Number	% Type I fibres	Type IIA fibres	Type IIB fibres	Type IIC fibres
20–29	26	7	39.1±3.4 (15–56)	34.0±2.8 (27–41)	24.3±4.1 (15–43)	2.7±0.6 (1 5)
30–39	36	11	36.1±1.6 (28–44)	38.6±2.4 (26–51)	22.1±2.1 (14–34)	4.0±1.2 (1 11)
40–49	43	7	49.6±5.2 (37 75)	33.6±3.1 (22–47)	15.0±4.5 (2 28)	1.9±0.9 (0–7)
50–59	55	9	49.4±5.1 (23–72)	28.7±2.7 (14–40)	19.4±3.9 (4–37)	1.6±0.6 (0–5)
60–65	62	7	65.6±4.8 (46–85)	19.6±2.1 (13–29)	12.9±4.3 (1 36)	2.3±0.6 (1–4

Values are means ± S.E. Values in parentheses are highest and lowest observations.

10–12. The subjects were employees of an insurance company (clerks) and were untrained to moderately trained (group I II according to Saltin and Grimby (1968) in respect to occupational and spare time physical activity).

Muscle biopsies were obtained from the left vastus lateralis muscle using the needle biopsy technique (Bergström 1962). Biopsies for enzyme activity analyses were immediately frozen in liquid nitrogen and stored at −80°C. Enzyme activity was determined by fluorometric means using NAD-NADP coupled reactions according to Lowry and Passonneau (1972) The enzymes investigated were Mg^{2+} stimulated ATPase (E.C. 3.6.1.4), myokinase (MK) (E.C. 2.7.4.3) and lactate dehydrogenase (LDH) (E.C. 1.1.1.27). The LDH isozymes in muscle samples were separated by means of disc-electrophoresis and stained. The intensity of stained bands corresponding to the LDH isozymes was quantified by means of densitometric scanning according to Penney *et al* (1974). The relative quantity of M and H subunits was the calculated according to Thorling and Jensen (1966). Biopsies for histochemical analyses were transversely mounted frozen in isopentane cooled with liquid nitrogen and stored at −80°C until analysis.

Serial transverse sections (10 μm) were cut with a microtome at −20°C and stained for myofibrillar ATPase (Gomori 1941 Padykula and Herman 1955) and for NADH-diaphorase activity (Novikoff *et al* 1961). Photographs of the stained sections were taken and fibre classification into type I and II (Engel 1962) was made in all 55 subjects. In 41 of the subjects the type II fibres were subclassified into A, B and C subgroups (Brooke and Kaiser 1970 Dubowitz and Brooke 1973). No significant difference in percentage distribution of type I and type II fibres was found between the groups consisting of 41 and 55 subjects, respectively The mean number of counted fibres for classification into main groups (type I and type II) and subgroups (type I type IIA, B and C) was 602 ± 52 and 265 ± 32, respectively Fibre area determination was made from the photograph of the NADH-diaphorase staining via a cutting and weighing technique (for detailed information see Thorstensson *et al* 1977)

Ordinary statistical methods were used to calculate means and standard error of the means (S.E.). The significance of linear correlation coefficients was tested from individual values according to Snedecor and Cochran (1967)

Results

The average muscle fibre type distribution, fibre area and relative fibre area occupied by each fibre type in the muscle are shown in Table I and Table II Fibre type distribution data for all 55 subjects displayed a significant ($p < 0.001$) linear increase in the percentage of type I fibres with increasing age (Fig. 1) Thus, the average type I fibre distribution was 55% in the 60–65 year old group compared with 41% in the 20–29 year old group. Type II fibre subclassification revealed no significant change in type IIC fibre distribution (ranged 2–4%), whereas the percentile distribution of type IIA and type IIB both decreased significantly ($p < 0.001$ and $p < 0.05$ respectively). Type IIA averaged 34% in the 20–29 year old group as compared to 20% in the 60–65 year old group The type IIB/IIA fibre ratio did not, on the other hand change with age, although a tendency to an increase was observed Neither could any significant difference be observed between means of the two youngest groups as compared with means of the two oldest groups.

When the individual data were taken together type IIB/IIA fibre ratio demonstrated an inverse relationship versus type I fibre distribution (r 0.55 $p < 0.001$). This pattern was also true for all age groups studied although significant only for some groups. This means that a subject irrespective of age with a high percent of type I fibre in his skeletal muscle, simultaneously had a relatively lower proportion of type IIB fibres.

The fibre area determinations revealed a selective linear decrease in type II fibre area ($p < 0.01$), while no significant change in type I fibre area could be seen The type II fibre area decreased from 6 853 μm in the 20–29 year old group to 5 243 μm in the 60–65 year old group This corresponds to a 33% decrease in absolute type II fibre area. The type II/I

TABLE III. Enzyme activities of Mg^{2+} stimulated ATPase, myokinase and lactate dehydrogenase (LDH). Lactate dehydrogenase isozyme distribution expressed as enzyme activity of H-LDH and M-LDH and as M-LDH % of LDH total.

Group	Mean age yr	Number	Mg^{++} stim ATPase 10^{-6} moles g^{-1} min^{-1}	Myokinase 10^{-6} moles g^{-1} min^{-1}	Lactate dehydrogenase (LDH) 10^{-4} moles g^{-1} min^{-1} PA→LA	LA→PA	H-LDH	M-LDH	% M-LDH of total LDH
20–29	26	11	11.7±1.3 (6.9–20.0)	146.4±7.2 (108.2–179.0)	0.43±0.06 (0.24–0.85)	0.51±0.06 (0.32–0.84)	0.10±0.02 (0.03–0.20)	0.32±0.05 (0.16–0.65)	74.0±3.1 (60–94)
30–39	36	12	10.8±1.3 (6.4–19.5)	152.9±12.1 (110.9–238.4)	0.59±0.07 (0.16–0.95)	0.64±0.07 (0.23–0.93)	0.15±0.03 (0.04–0.45)	0.46±0.06 (0.06–0.69)	70.9±4.7 (45–91)
40–49	45	10	11.5±1.3 (5.5–18.8)	133.5±13.0 (69.3–352.3)	0.42±0.06 (0.11–0.78)	0.50±0.07 (0.15–0.82)	0.16±0.02 (0.08–0.29)	0.26±0.05 (0.03–0.49)	65.4±2.4 (54–77)
50–59	55	12	9.9±0.7 (5.8–13.3)	141.1±15.3 (40.3–235.9)	0.48±0.05 (0.17–0.90)	0.47±0.05 (0.11–0.81)	0.16±0.03 (0.04–0.34)	0.27±0.07 (0.08–0.78)	63.7±5.0 (44–88)
60–65	62	10	12.2±1.0 (9.7–18.5)	142.1±5.8 (114.7–171.6)	0.38±0.05 (0.20–0.77)	0.44±0.06 (0.29–0.84)	0.16±0.03 (0.07–0.32)	0.23±0.03 (0.13–0.41)	59.1±3.3 (45–67)

Values are means ± S.E. Values in parentheses are highest and lowest observations.

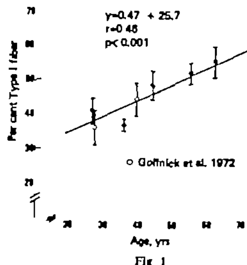


Fig 1

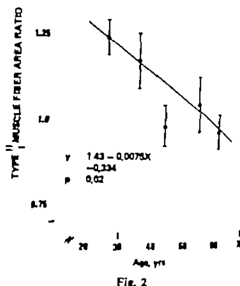


Fig. 2

Fig. 1 Percentage distribution of type I fibres (vastus lateralis muscle) versus age. Mean values ± S.E. are shown along with the regression line. Regression line is calculated from individual values. Data from Gollnick *et al* (1972) are also plotted.

Fig. 2. Type II fibre area ratio versus age. Mean values ± S.E. are shown along with the regression line. Regression line is calculated from individual values.

Discussion

The main finding in the present study was the shift in fibre type distribution towards a higher percentage in type I fibres and a corresponding decrease in type II fibres with age. The percentage distribution of type IIA and type IIB fibres also decreased, while type IIB/IIA fibre ratio and type IIC fibres did not change significantly. Moreover the type IIB/IIA fibre ratio was found to be inversely related to percentage type I fibres. A selective decrease in type II fibre area was found with age, while type I fibre area did not change significantly. The enzyme activity of Mg^{2+} stimulated ATPase and myokinase remained unchanged, while the activity of lactate dehydrogenase (LDH) was found to be higher in the youngest groups as compared with the oldest. LDH isozyme pattern shifted towards a decrease in percentage distribution of the muscle-specific isozymes and a corresponding decrease in activity while the activity of the heart-specific isozymes did not change.

The subjects in the present study were recruited on the basis of an equal occupational and spare time physical activity level (Saltin and Grimby 1968) to get a population as homogeneous as possible. However it cannot be ruled out that the subjects adapted to a more sedentary way of living with age, although having almost equal occupational and spare time physical activity levels. Irrespective whether this is an endogenous or a sociological-anthropological process, it must be regarded as a part of the overall ageing process with its significance for muscle tissue adaptation.

No systematical studies in healthy men at age 22 to 65 years are available. Gollnick *et al*

anterior muscle of the rat, Tauchi *et al* (1971) have claimed that there is a decrease in the number and volume of type II fibres and in the number of type I fibres. This selective atrophy of type II fibres is in accordance with findings in the old rat soleus muscle (Bass *et al* 1975).

According to the animal studies quoted above, it seems likely that there is an increase in type II fibre area and a transformation of type II fibres into type I fibres during maturation, while in the ageing muscle there is a decrease in type II fibre area and a loss of muscle fibres. Whether the change in fibre type distribution taking place in the ageing muscle in the present study is due to a transformation of type II fibres cannot be answered, since the total number of muscle fibres have not been determined. The selective type II fibre atrophy seen in the ageing muscle also occurs in conditions such as denervation, inactivity and malnutrition (Engel 1965, Engel 1970).

Findings by Gutmann and Hanzlíková (1966) have shown that the metabolic profile of the muscles of an old animal resembles denervated muscles. The preferential atrophy of type II fibres in denervated muscles (Engel 1962) has been suggested to be due to a preferential susceptibility of type II fibres to loss of trophic influence from both type I and type II neurons (Karpati and Engel 1968, Engel 1970, Engel and Warmolts 1971). However human muscle fibre types differ in many respects from those of cat and rat (Schmalbruch and Kamieniecka 1974). This must be taken into account when comparing the information available from animal and human studies. As has been pointed out above, it cannot be ruled out that the activity level decreases with age although the subjects have an almost equal occupational and spare time physical activity. This might cause the selective type II fibre atrophy. However, at the present time it is impossible to evaluate whether the type II fibre atrophy is related to denervation/inactivity or both.

The changes found in LDH activity isozyme pattern and isozyme activity are in accordance with findings in fibre type distribution observed in the present study (Karlsson *et al.* 1974). However, it cannot be established whether the shift in isozyme activity is due to a transformation of type II fibres into type I fibres or a degeneration of type II fibres, since the activity in the whole muscle has not been measured.

The metabolic changes found in the ageing human muscle, *i.e.* an increasing percentage of type I fibres, a selective type II fibre atrophy and a decrease in the activity of the muscle specific LDH isozymes, might explain some of the changes found in physical capacity with age, such as increasing slowness of movement and decreasing muscular strength. Which of the underlying mechanisms may be causing these changes can only be speculated upon. According to data available so far from human and animal studies, the most probable cause for these metabolic changes is an atrophy and degeneration of the type II fibres rather than a transformation of the type II fibres. This degeneration of the type II fibres might be due to decreasing trophic influence from the motoneurons caused by a denervation-like process.

This study was supported by grants from the Swedish Medical Research Council (B77/78-04X-4251-04A), the Research Council of the Swedish Sports Federation, Svenska sällskapet för medicinsk forskning, and Skandia Insurance Company.

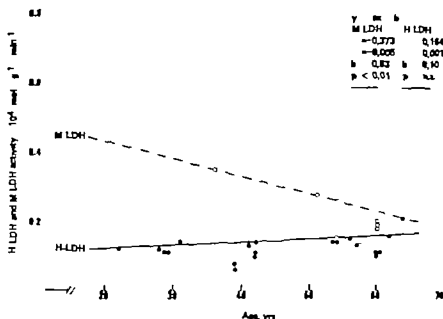


Fig. 3 M LDH and H LDH activity versus age, respectively Individual values are shown along with regression lines.

(1972) compared muscle fibre type distribution in physically untrained and trained men. Their study provided data on the fibre type distribution in untrained men in two age groups, 24–30 and 31–52 years The percentage of type I fibres amounted to 36 and 44 %, respectively The present data are in good agreement with Gollnick *et al* 1972 (Fig. 1).

A recently published study (Tomonaga 1977), using histochemical and electron microscopic methods studied skeletal muscles in 79 elderly patients (mainly in the 60-90 age group). The most prominent findings in the senile muscle were the neuropathic changes (group atrophy small dark angulated fibres, target fibres, type grouping and nuclear clumps) and type II fibre atrophy It was also found that type II fibre atrophy was more common in the proximal muscles of the lower extremity than neuropathic changes, whereas the reverse relationship was found in the distal muscles of the lower extremity Type II fibre area decreased ($p < 0.05$) with age while type I fibre area remained unchanged Consequently the type II/I fibre area ratio decreased ($p < 0.01$), a circumstance in agreement with findings in the present study

Studies on rat soleus muscle during growth (Kugelberg 1976) have shown an increasing percentage of type I fibres with age and a corresponding decrease in the percentage of type II fibres. Similar data were obtained by Karpati and Engel (1967) during maturation in guinea pig, rat and cat soleus muscle. The latter authors suggested this to be due to a transformation of type II fibres into type I rather than atrophy or degeneration of the type II fibres, since the total number of fibres increased and the diameter of the type II fibre gradually increased until they disappeared. According to Kugelberg, the transformation is due to changing properties of the motoneurons, *i e* from phasic to tonic. Other studies (Gutmann and Hanzlíková 1966, Bass *et al* 1975) examining the old rat soleus muscle have reported a loss of muscle fibres and a decrease in fibre size in the soleus muscle. On the basis of percentage changes in type I fibres and decrease in number of fibres in the senescent

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enzymes, i.e. phosphofructokinase (E.C.2.7.1.11.), glycogen phosphorylase (E.C.2.4.1.1.) and lactate dehydrogenase ((LDH) E.C.1.1.1.27.), in the two main muscle fibre types (Essén *et al.* 1975, Sjödin 1976, Thorstensson 1976). Thus, both LDH activity and the LDH isozyme distribution pattern in an untrained or moderately trained human population were found to be related to the percentage of FT fibres in the muscle (Karlsson *et al.* 1974). A muscle rich in FT fibres displayed greater LDH activity and a larger contribution of muscle-specific LDH (M-LDH) than a muscle rich in ST fibres in which the heart-specific type of LDH (H-LDH) is predominant. The intracellular distribution of these two main LDH isozymes and their kinetic properties differ (for ref. see Sjödin 1976). In normal conditions, the M-LDH isozymes are mainly associated with membranes, such as the sarcoplasmic reticulum. On the other hand, H-LDH fractions have been found in the inner membrane of the mitochondria.

The aim of the present investigation was to study lactate accumulation in the muscles of individuals differing with respect to certain relevant muscle qualities. Therefore, muscle lactate concentration was determined after intense, standardized exercise of brief duration and related to individual muscle fibre type distribution and LDH characteristics.

Subjects, Procedures and Methods

10 male physical education students served as subjects. Their mean ($\pm$ S.E.) age, height and weight were 24 ± 1 yrs, 179 ± 1 cm and 71.3 ± 2.1 kg respectively. Muscle biopsies (Bergström 1962) were taken from the vastus lateralis of the left leg at rest. Histochemical staining for myofibrillar ATPase was undertaken after preincubation at pH 10.3, 4.6 and 4.3 according to Brooke and Kaiser (1970) in order to classify muscle fibres as fast twitch (FT) and slow twitch (ST) fibres respectively and in order to separate the FT fibres into FTa and FTb subgroups. Muscle fibre area was determined according to Thorstensson (1976) from transverse muscle sections stained for NADH diaphorase activity according to Novikoff *et al.* (1961).

The subjects performed 25 repeated maximal isokinetic leg extensions at a velocity corresponding to 3.14 rad · s^{-1} so as to induce substantial local muscle fatigue (Thorstensson 1976). The exercise was performed with the subject seated in a fixed position with his leg attached to the lever arm of an isokinetic dynamometer (Cybex II, Lumex Inc., New York) as described elsewhere (Thorstensson 1976). During this type of exercise, EMG recordings have shown that both medial and lateral parts of the vastus muscle are heavily activated (Merrifield and Dostal 1977). In order to ensure that subjects actually exerted maximal force, 3 single leg extensions were performed a few minutes prior to the experiment. The mean force exerted when a single extension was performed amounted to 161.6 Newtonmeter (Nm) compared to 160.4 Nm in the experimental situation.

Immediately after (within 3–4 s) the 25th leg extension, a muscle biopsy was taken from the vastus lateralis, frozen in liquid nitrogen and stored at −80°C until analysed. Muscle biopsies were then freeze-dried, and approximately 100 fibres were dissected out (21°C and 30% humidity) and separated into FT and ST fibres on the basis of myofibrillar ATPase staining following preincubation at pH 10.3 according to Essén *et al.* (1975). Pooled ST and FT fibres respectively consisting of 5–10 fibre fragments per pool were weighed on a Cahn electro-balance (Karlsson 1971). The samples ranged 0.01–0.03 mg in weight. The lactate concentration was determined by fluorometric means (Karlsson 1971). Each value was then calculated as a mean value for the lactate concentration in 2–7 pools of the respective fibre type. All values were converted into wet weight values. A water content of 77% was assumed for muscle biopsy specimens (Karlsson 1971).

Additional dissected pools of FT and ST fibres consisting of about 100 fibres each were homogenized in 100 µl of 0.5 M KCl. Total LDH activity in the forward reaction (pyruvate→lactate, LDH_{tot}) in the homogenate, diluted 1:4 with 0.1 M Tris-HCl buffer pH 7.5, was determined by fluorometric means according to Lowry and Passonneau (1972). Discelectrophoresis for separating the LDH isozymes was performed according to Dietz and Lubrano (1967) on 25 µl of the homogenate. The relative contribution of muscle-specific LDH isozymes (% M-LDH) was determined using a densitometric scanning technique on the separated and stained LDH isozyme bands in the gels according to Sjödin (1976). The activity corresponding to the muscle-specific LDH (M-LDH) was then calculated (% M-LDH × LDH_{tot}).

Acta physiol scand. 1978. 103 40–46

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Relationship between lactate accumulation, LDH activity, LDH isozyme and fibre type distribution in human skeletal muscle

By

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Received 31 October 1977

Abstract

TESCH, P., B SJÖDIN and J KARLSSON *Relationship between lactate accumulation, LDH activity LDH isozyme and fibre type distribution in human skeletal muscle* Acta physiol. scand. 1978 *103* 40–46.

Lactat concentration total LDH activity and muscle-specific LDH isozymes were determined in pools of the two main types of human skeletal muscle fibres. Analyses were made from biopsy specimens obtained after intense dynamic exercise lasting approximately 30 s. Lactate concentration, total LDH activity and muscle-specific LDH activity displayed higher average values for FT (fast twitch) f bres tha for ST (slow twitch) fibres. In addition, positive correlations were found both between the individual percentage of FT f bres and muscle lactate concentration nd between lactate concentration and total LDH activity and muscl -specific LDH activity respect vely

High lactate levels have been demonstrated both after exhaustive isometric exercise (Ahlborg *et al* 1972, Karlsson and Ollander 1972) and maximal dynamic exercise (Karlsson 1971 Knuttgen and Saltin 1972) of brief duration and have been suggested as one possible factor contributing to muscle fatigue in maximal exercise of brief duration

When the lactate concentration in different parts of a muscle sample were analysed after maximal exercise, values in the contracting elements were found to be 3–5 times greater than in elements supposed to be non-contracting (Karlsson 1971) Therefore, it may be assumed that the concentration of metabolites found in whole muscle biopsy specimens does not always reflect the true state of lactate accumulation in exercised muscle.

Essén and Häggmark (1975) reported a greater accumulation of lactate in type II (fast twitch FT) muscle fibres than in type I (slow twitch, ST) muscle fibres after exhaustive isometric exercise Recent studies of lactate accumulation in different single muscle fibres have indicated that the fibre type distribution of a muscle is important to lactate accumulation in single muscle fibres (Tesch and Karlsson 1977) In other words, a muscle rich in FT fibres displays a greater potential for accumulating lactate than a muscle rich in ST fibres. This difference may be due to differences in the activity of the glycogenolytic system s regulating

enzymes, i.e. phosphofructokinase (E.C.2.7.1.11), glycogen phosphorylase (E.C.2.4.1.1) and lactate dehydrogenase ((LDH) E.C.1.1.1.27.), in the two main muscle fibre types (Essén et al. 1975, Sjödin 1976, Thorstensson 1976). Thus, both LDH activity and the LDH isozyme distribution pattern in an untrained or moderately trained human population were found to be related to the percentage of FT fibres in the muscle (Karlsson et al. 1974). A muscle rich in FT fibres displayed greater LDH activity and a larger contribution of muscle-specific LDH (M-LDH) than a muscle rich in ST fibres in which the heart-specific type of LDH (H-LDH) was predominant. The intracellular distribution of these two main LDH isozymes and their kinetic properties differ (for ref. see Sjödin 1976). In normal conditions, the M-LDH isozymes are mainly associated with membranes, such as the sarcoplasmic reticulum. On the other hand, H-LDH fractions have been found in the inner membrane of the mitochondria.

The aim of the present investigation was to study lactate accumulation in the muscles of individuals differing with respect to certain relevant muscle qualities. Therefore, muscle lactate concentration was determined after intense, standardized exercise of brief duration and related to individual muscle fibre type distribution and LDH characteristics.

Subjects, Procedure and Methods

10 male physical education students served as subjects. Their mean ($\pm$ S.E.) age, height and weight were 4 1 yrs, 179 1 cm and 71.1$\pm$2.1 kg respectively. Muscle biopsies (Bergström 1962) were taken from the vastus lateralis of the left leg at rest. Histochemical staining for myofibrillar ATPase was undertaken after preincubation at pH 10.3, 4.6 and 4.3 according to Brooke and Kaiser (1970) in order to classify muscle fibres as fast twitch (FT) and slow twitch (ST) fibres respectively and in order to separate the FT fibres into FTa and FTb subgroups. Muscle fibre area was determined according to Thorstensson (1976) from transverse muscle sections stained for NADH diaphorase activity according to Novikoff et al. (1961).

The subjects performed 25 repeated maximal isokinetic leg extensions at a velocity corresponding to 3.14 rad s^{-1} so as to induce substantial local muscle fatigue (Thorstensson 1976). The exercise was performed with the subject seated in a fixed position with his leg attached to the lever arm of an isokinetic dynamometer (Cybex II, Lumex Inc. New York) as described elsewhere (Thorstensson 1976). During this type of exercise, EMG recordings have shown that both medial and lateral parts of the vastus muscle are heavily activated (Mernfield and Dostal 1977). In order to ensure that subjects actually exerted maximal force, 3 single leg extensions were performed a few minutes prior to the experiment. The mean force exerted when single extension was performed amounted to 161.6 Newtonmeter (Nm) compared to 160.4 Nm in the experimental situation.

Immediately after (within 3–4 s) the 25th leg extension, a muscle biopsy was taken from the vastus lateralis, frozen in liquid nitrogen and stored at –80°C until analysed. Muscle biopsies were then freeze-dried, and approximately 100 fibres were dissected out (21°C and 30 % humidity) and separated into FT and ST fibres on the basis of myofibrillar ATPase staining following preincubation at pH 10.3 according to Essén et al. (1975). Pooled ST and FT fibres respectively consisting of 5–10 fibre fragments per pool were weighed on a Cahn electro-balance (Karlsson 1971). The samples ranged 0.01–0.03 mg in weight. The lactate concentration was determined by fluorometric means (Karlsson 1971). Each value was then calculated as a mean value for the lactate concentration in 2–7 pools of the respective fibre type. All values were converted into wet weight values. A water content of 77 % was assumed for muscle biopsy specimens (Karlsson 1971).

Additional dissected pools of FT and ST fibres consisting of about 100 fibres each were homogenized in 300 μl of 0.5 M KCl. Total LDH activity in the forward reaction (pyruvate → lactate, LDH_{tot}) in the homogenate, diluted 1:4 with 0.1 M Tris-HCl buffer pH 7.5, was determined by fluorometric means according to Lowry and Passonneau (1972). Discelectrophoresis for separating the LDH isozymes was performed according to Dietz and Lubrano (1967) on 25 μl of the homogenate. The relative contribution of muscle-specific LDH monomers (% M-LDH) was determined using a densitometric scanning technique on the separated and stained LDH isozyme bands in the gels according to Sjödin (1976). The activity corresponding to the muscle-specific LDH (M-LDH) was then calculated (% M-LDH · LDH_{tot}).

TABLE I Mean values and range for lactate concentration, total LDH activity and M-LDH activity in different muscle fibre types and whole muscle respectively

	FT fibres		ST fibres			Whole muscle	
	Mean	Range	Mean	Range	Difference	Mean	Range
Lactate concentration mmol · kg⁻ wet weight	21 7	(4.0–31.3)	15.0	(3 4–30.5)	$p<0.005$	18.5	(3.5–31.1)
LDH_{tot} activity × mmol · min⁻ × kg⁻ wet weight · 10⁴	1 12	(0.52–1 82)	0.61	(0.33–1 11)	$p<0.001$	0.90	(0.38–1 63)
M LDH activity × mmol · min⁻¹ × kg⁻ wet weight × 10⁴	0.82	(0.37–1 76)	0.28	(0.10–0.81)	$p<0.001$	0.60	(0.18–1.50)

The following formula was used in order to express lactate concentration for the entire muscle investigated Lactate concentration in FT fibres · % FT area + lactate concentration in ST fibres · % ST area. LDH activity or M LDH activity were calculated in the corresponding manner by substituting lactate content for LDH activity in the two main fibre types.

Results

The average percentage of FT fibres in the studied subjects was 46.5% FT fibres (range 29–74%) When the FT fibres were subdivided into FTa and FTb fibre groups, the mean values were found to be 28 and 15% respectively of the total number of fibres. 3 per cent of the fibres were classified as unidentified The mean relative area taken up by FT fibres amounted to 62.0% (range 28–76%)

Lactate concentration after exercise averaged 21 7 and 15 0 mmol · kg⁻ wet muscle in FT fibres and ST fibres respectively There was a considerable inter individual spread in terms of the lactate concentration in both FT (4 0–31.3 mmol · kg⁻) and ST fibres (3 4–30.5 mmol · kg⁻) (Table I) The lowest lactate concentration, irrespective of muscle fibre type, was observed in the subject with a muscle richest in ST fibres, whereas the highest lactate concentration observed was found in a muscle consisting of predominantly FT fibres.

The whole muscle values calculated for lactate concentration, LDH_{tot} and M LDH activity

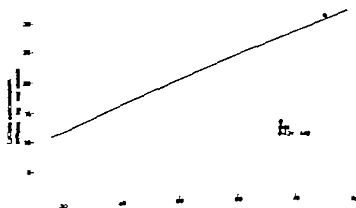


Fig. 1 The relationship between muscle lactate concentration (lactate concentration in FT fibres · % FT area + lactate concentration in ST fibres · % ST area) and individual muscle fibre type distribution (% FT).

TABLE II. Correlation coefficients and levels of significance for some variables investigated.

	Correlation coefficient	Level of significance
Lactate concentration in muscle—muscle fibre type distribution	−0.74	$p < 0.01$
Lactate content in FT fibres—muscle fibre type distribution	−0.91	p 0.001
Lactate content in ST fibres—muscle fibre type distribution	−−0.04	n.s.
Lactate concentration in muscle—LDH_{tot} activity	0.66	$p < 0.05$
Lactate concentration in muscle—M-LDH activity	0.70	p 0.02
LDH_{tot} activity—muscle fibre type distribution	0.96	p 0.001
M-LDH activity—muscle fibre type distribution	−0.98	p 0.001
M-LDH in FT fibres—muscle fibre type distribution	0.74	$p < 0.01$
M LDH in ST fibres—muscle fibre type distribution	−0.89	p 0.001

were 18.5 (range 3.5–31.1) mmol · kg^{-1} 0.90 (range 0.38–1.65) and 0.60 (range 0.18–1.54) mmol · kg^{-1} · min · 10^{-4} wet weight (Table I).

Positive correlations were present between individual muscle lactate concentrations and individual muscle fibre type distribution (Fig. 1 Table II) as well as between individual lactate concentration and individual LDH_{tot} activity and M LDH activity (Fig. 2, Table II). In agreement with earlier studies (Karlsson *et al.* 1974), positive correlations were found for individual LDH_{tot} activity and M-LDH activity erus individual fibre type distribution (Table II). The relative distribution of M-LDH in both FT and ST fibres was also correlated to the individual muscle fibre type distribution (Fig. 3, Table II).

No significant differences in lactate accumulation were found in subjects with approximately the same percentage of FT fibres but a different FTa/FTb ratio.

Lactate concentration (mmol · kg^{-1} wet weight) in FT fibres % FT area correlated strongly to % FT whereas no corresponding correlation was found between lactate concentration in ST fibres · % ST area and % FT (Fig. 4 Table II).

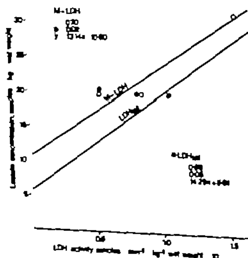


Fig. 2. The relationship between muscle lactate concentration and LDH_{tot} and M-LDH activity respectively.

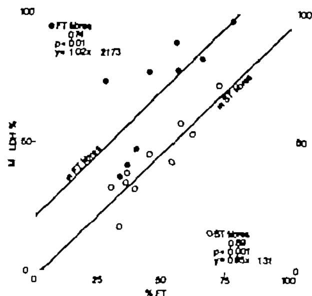


Fig. 3 The relative distribution of M-LDH in FT and ST fibres respectively in relation to individual muscle fibre type distribution (% FT).

Discussion

In the present study a considerable accumulation of lactate was observed in the exercised muscle after only about 30 s. The lactate was not uniformly distributed in the entire exercised muscles, as has been noted elsewhere (Karlsson 1971) The results agree with data obtained from expts. on muscles with different muscle fibre types in rat (Beatty *et al* 1963). As demonstrated in Fig. 4 there was a positive relationship between the lactate content in the FT fibre portion of the muscle and the percentage of FT fibres in the whole exercised muscle. Corresponding negative relationship did not exist for the lactate content in the ST fibre portion and the percentage of FT fibres, which could be expected assuming that the different fibre types form the same amounts of lactate.

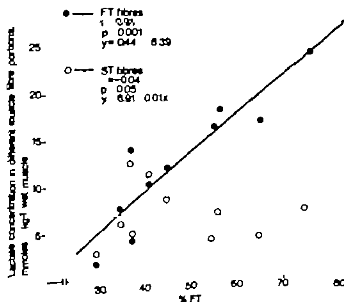


Fig. 4 Lactate content in FT (lactate concentration in FT fibres FT area) and ST (lactate concentration in ST fibres ST area) fibre portions of the muscles respectively in relation to individual muscle fibre type distribution (% FT).

The individual differences with respect to lactate accumulated in the muscles after exercise are found to be related to individual variations in percentage of FT fibres, total LDH activity and LDH isozyme pattern. Thus, muscles which had produced high lactate concentrations displayed higher percentage of FT muscle fibres and had a high total LDH and M-LDH activity. The M-LDH isozymes have a higher specific activity and are known to be more efficient in the lactate formation processes as compared to the H LDH isozymes.

From training studies (Sjödin *et al.* 1976) and from studies on tissue cultures (Dawson *et al.* 1964) it might be established that the conditions in the entire muscle, aerobic or anaerobic, will influence the LDH isozyme pattern in the single muscle cells. Aerobic conditions have been shown to inhibit the M-LDH subunit production while the opposite was true during anaerobic conditions. Due to a lower capillary density in a muscle rich in FT fibres (Andersen, 1975), the conditions have to be more anaerobic than in a muscle rich in ST fibres. Therefore, it was not surprising findings that the relative M LDH activity in single FT and ST fibres respectively was related to the percentage of FT fibres in the different muscles examined (Fig. 3).

Thus, the consistent pattern with higher lactate concentrations in FT fibres than in ST fibres may be due to the different metabolic profiles of the two main muscle fibre types (Gollnick *et al.* 1974). Support for this view was provided by the present LDH and lactate data.

No lactate analyses were performed on the different subgroups of FT fibres. Consequently it is impossible to determine whether the lactate concentration was more pronounced in one FT subgroup or the other. On the basis of differences in enzymatic profiles, the FTb fibre type might be expected to be better of forming lactate, as noted by Essén *et al.* (1975). However no support for any such assumption was found when subjects with similar muscle fibre type distribution but different FTa/FTb ratios were compared.

The strong correlation between individual muscle fibre type distribution, expressed as percentage of FT fibres, and both total LDH activity and the relative contribution of M LDH as found in previous studies (Sjödin 1976) was confirmed. Differences observed in total LDH activity, M-LDH and H LDH respectively between the two main muscle fibre types also confirmed previous findings (Sjödin 1976).

In summarizing, data obtained in the present study confirmed previous findings indicating different metabolic properties for the two main skeletal muscle fibres. Thus, fast twitch muscle fibres, characterized by great LDH_{tot} activity and a high proportion of M-LDH are better at forming lactate than slow twitch muscle fibres.

This study was supported by grants from the Swedish Medical Research Council (B77-04X-4251-04A, B78-04X-4251-05B) and the Research Council of the Swedish Sports Federation.

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Acta physiol. scand. 1978, 103, 47–51

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Isometric strength performance and muscle fibre type distribution in man

By

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Received 31 October 1977

Abstract

TESCH, P and J KARLSSON. *Isometric strength performance and muscle fibre type distribution in man.* Acta physiol. scand. 1978, *103* 47–51

Maximal isometric one-leg strength (MIS) was determined in 31 physical education students displaying wide range in muscle fibre type distribution (21–79% fast twitch (FT) muscle fibres) in their vastus lateralis muscles. A linear positive correlation was found between *MIS* and the relative distribution of FT fibres ($p < 0.001$). This should mean that not only muscle mass involved but also the quality of muscle will be decisive for the ability to develop high isometric strength.

The maximal strength of a muscle has been shown to be proportional to the cross-sectional area of the muscle (Ikai and Fukunaga 1968). In accordance with this, some authors have reported that athletes trained for extreme strength, such as weight-lifters, displayed larger muscle fibres than untrained subjects (Edström and Ekblom 1972, Gollnick *et al.* 1972, Prince *et al.* 1976, Häggmark *et al.* 1978). Häggmark *et al.* also found a correlation between muscle fibre area and the muscle area determined by means of tomography. The "hypertrophy" was predominantly found in the fast twitch (FT or type II) muscle fibres. FT fibre hypertrophy was also noted after 8 weeks of strength training (Thorstensson 1976).

Animal experiments have indicated that white muscles are better adjusted to exerting force than red muscles, even during isometric conditions (Eccles and Sherrington 1930, Burke and Edgerton 1975).

EMG studies with human subjects have disclosed that fast twitch motor units are mainly recruited at high muscle tensions (Gydikov and Kosarov 1974, Burke and Edgerton 1975). Hultén *et al.* (1975) found a relationship between the individual relative percentage of slow twitch (ST) fibres and the duration of maximal isometric endurance in two-leg exercise, but they were unable to relate two-leg maximal isometric strength (MIS) to individual muscle fibre type distribution.

It was recently shown in humans that strength output during one-leg extension in a Cybex apparatus (Lumex Inc., New York) at a relatively fast rate of isokinetic muscle

TABLE I Anthropometric data, muscle fibre type distribution and muscle strength for the group n subgroup investigated Values are presented as mean ± S.E. nd range.

	Mean		±S.E.		Range	
	n = 31	n = 25	n = 31	n = 25	n = 31	n 25
Age	25	25	0.8	0.8	?0-38	20-35
Height	180	180	0 9	1 1	171 194	171 194
Weight	72	72	1 1	1.3	63-89	63-89
% FT	50	52	2.4	2.7	21 79	21 79
MIS, N	*1 160*	*1 185*	38.0	40 6	736-1 595	796-1 595
MIS, N kg⁻ b.w	16	16	0.5	0.5	11 22	12-22

contraction depends on the relative distribution of FT muscle fibres (Thorstensson 1976 He was unable to find any such relationship for either slow isokinetic contractions or isometric contractions. In view of this and the reported suggestion that the muscle fibre recruitment pattern may differ when one and two-leg exercise was compared (Secher *et al* 1977 the present investigation was undertaken in order to study one-leg MIS using the same experimental model as Hultén *et al* (1975)

Material and methods

31 male physical education students volunteered for this study All subjects were moderately trained to well-trained, but no one was regular participa t i strength- or sprint trainl g. Age, height and weight averaged 25 yrs, 180 cm and 72.3 kg respectively (Table I)

Biopsies (Bergström 1962) were obtained from the vastus lateralis muscle of the left leg. Muscle samples were mounted in embedding medi m, frozen in liquid nitrogen cooled isopentane and stored at 80 C unt l analysis. Cross-sections were histochemically stained for myof brillar ATPase after preincubation at pH 10.3 (Padyk la d Herman 1955). Muscle f bres were identified as slow twitch (ST) and fast twitch (FT) f bres respectively and the relative distribution of each f bre type was then calculated according to Gollnick *et al* (1972). *At least ?00 fibres were counted from each sample.*

Transverse serial sections were cut from specimens t ken from 25 of the subjects, stained for NADH d phorase according to Novikoff *et al* (1961) nd sed for calculation of muscle f bre area (Thorstensson 1976). Muscle biopsies were taken few days prio t stre gth measurements, made during maximal isometric leg extensions with the left knee t an gle corresponding t 90° Force was measured a strain gauge located on immovable iron ba under the subjects arches. The experimental setup has been described detail elsewhere (Karlsson and Ollander 1972, H ltén *et al.* 1975). Conventional statistical methods were employed t calculate mean values, standard error of the mea (S.E) and linear regression coefficient (r).

Results

Muscle fibre type distribution averaged 50.4% ± 2.4 (range 21–79) FT fibres in the present group (Table I) Mean maximal isometric strength (MIS) was 1160 Newton (N) or 16.1 N kg body weight (Table I) Practically the same values were obtained for strength performance and muscle fibre type distribution in the subgroup in which muscle fibre area was determined and relative FT fibre area calculated (Table I) The relative FT fibre area value obtained in this subgroup amounted to 57 0% ± 2.74 (range 28 77).

A correlation ($p < 0\,001$ and $p < 0\,01$ respectively) between either muscle fibre type distribution (% FT fibres) or relative muscle fibre area (% FT area) and one-leg MIS

MAXIMAL ISOMETRIC STRENGTH, N

1600
1200
800
0

$y = 8.8x + 725.8$
$r = 0.56$
$p < 0.001$

0 20 40 60 80

MUSCLE FIBRE DISTRIBUTION
(% FAST TWITCH FIBRES)

Fig. 1 The relationship between muscle fibre type distribution (% FT fibres) and maximal isometric one-leg strength (N).

and Table II) was found. When muscle strength was corrected for body weight, the correlation declined somewhat ($p < 0.01$ and $p < 0.05$ respectively) for both these relationships (Table II).

Discussion

On the basis of histochemical PAS stainings, Gollnick *et al.* (1974) and Secher and Nygaard-Jensen (1976) suggested that FT fibres are mainly recruited during maximal isometric voluntary contractions. The circumstance that FT fibres are activated at high relative isometric tensions, also demonstrated by Gydikov and Kosarov (1974), leads to the hypothesis that a muscle predominantly consisting of FT fibres is better adapted to the development of high tensions than a muscle rich in ST fibres.

The present results appear to confirm the hypothesis. However, the results failed to agree with the findings in a previous study based on the same experimental protocol but performed with two-leg exercise (Hultén *et al.* 1975). By way of contrast, Komi *et al.* (1977) were successful in correlating two-leg MIS to the relative distribution of FT fibres in top athletes, including athletes trained for strength and endurance. However, these two athletic extremes did differ in respect to muscle fibre type distribution. Thus, athletes representing "power events" were characterized by a high proportion of FT muscle fibres, whereas the muscles of endurance athletes were made up of predominantly ST fibres. However, the

TABLE II Correlation coefficients for variables of significance.

[illegible]	[illegible]	Correlation coefficient	Level of significance
MIS, N vs % FT	31	0.55	$p < 0.001$
MIS, N · kg^{-1} b.w. vs % FT	31	0.46	$p < 0.01$
MIS, N vs % FT area	25	0.58	$p < 0.01$
MIS, N · kg^{-1} b.w. vs % FT area	25	0.49	$p < 0.05$

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A correlation (p < 0 001 and p < 0 01 respectively) between either muscle fibre type distribution (% FT fibres) or relative muscle fibre area (% FT area) and one-leg MIS (Fig. 1

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striking difference in the training regimens practiced by these two groups of athletes obscured the relationship between two-leg MIS and individual muscle fibre type distribution The interpretation of findings is consequently not conclusive.

In addition, information is available pointing to reduced muscle fibre involvement in two-leg exercise compared to one-leg exercise (Secher *et al* 1977). On the basis of results obtained from experiments in which FT motor units were partially blocked by decamethonium, these authors suggested that fibre recruitment was restricted in two-leg exercise compared to one leg exercise.

When one-leg and two-leg MIS were compared in successful and less successful oarsmen respectively Secher (1975) found that the latter group was incapable of developing twice the one leg force" during two-leg exercise in contrast to the ability of the successful oarsmen The same pattern was found when body builders and non-strength trained subjects were compared (unpublished observations)

One plausible explanation for this phenomenon is that neuromotor training effects are involved leading to an increased ability to recruit motor units during two-leg exercise It seems relevant to point out from Secher s two studies that there is some kind of neuromotor blockade during two-leg muscle contraction by untrained subjects. This blockade decreases with strength training. Data obtained by Komi and co-workers (1977) must be interpreted against this background *i.e* their data need not necessarily indicate that subjects rich in FT muscle fibres are stronger

However the present data on one leg MIS determinations do lend further support to the view that subjects rich in FT fibres are isometrically stronger The reason why Thorstensson (1976) was unable to reach the same conclusion is not clear at the moment but may have been related to the different experimental procedure applied.

The greater percentage of FT fibres which was found in the muscles of top athletes participating in "power events" might be the result of selective processes based on the aforementioned relationships between strength performance and muscle fibre type distribution

To summarize, data were obtained suggesting that muscle fibre type distribution is of importance even to maximal isometric strength performance capacity in addition to its significance in isometric endurance and relatively fast dynamic contractions as previously reported.

This study was supported by grants from the Swed sh Medical Research Council (B77-04X-4251-04A, B78-04X-4251-05B) nd the Research Council of the Swed sh Sports Federation.

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The present investigation was undertaken in order to elucidate whether or not beta blockers, when given simultaneously with thyroxine, could antagonize the thyroxine induced metabolic changes in heart or skeletal muscle (Kubista *et al.* 1971) or in brown adipose tissue (LeBlanc and Villemaire 1970, Ricquier *et al.* 1976, Rabi and Cassuto 1976). The ability of prolonged beta blockade to reduce the enhanced heat production capacity which results from thyroxine injections, was tested by measuring the heat loss of swimming rats (Dawson *et al.* 1970) or the calorigenic response to injected noradrenaline (LeBlanc and Villemaire 1970).

Material and methods

Adult male wistar rats of the Wistar/Af/Han/(Han 67) strain with an average weight of 200 g at the beginning of the experiment were used in these studies. The first group was composed of 18 control animals, injected daily with 0.5 ml of physiological saline-glycerol mixture (1 : 2); the second group of 14 rats injected daily with 50 µg 1-thyroxine/kg, the third group of 4 rats injected daily with 20 mg alprenolol/kg, and finally the fourth group composed of 15 rats, injected daily with 50 µg 1-thyroxine + 20 mg alprenolol/kg. The drugs were dissolved in physiological saline and were injected in saline-glycerol (1 : 2) mixture subcutaneously. The injections were given five times per week for 5 weeks. The animals were kept at room temperature (20–21 °C) during the drug regime. 24 h after the 20th drug injection the colonic temperature response to noradrenaline (0.5 mg/kg i.p.) was determined at thermoneutral temperature of 29 °C (LeBlanc and Villemaire 1970). Furthermore, the cooling rate of the rats when swimming in water at 25 °C was measured (Dawson *et al.* 1970). Colonic temperatures were obtained with thermocouple inserted to depth of 4 cm, and recorded on an Elab TE 3 (Copenhagen) potentiometer. This test was performed 24 h after the 22nd drug injection.

The rats were killed 24 h after the last injection by decapitation. Heart, adrenals and interscapular brown adipose tissue (ISBAT) were removed and weighed. Samples of the tip of the myocardium, of the thigh (M. vastus lateralis) muscle and of the ISBAT were immediately frozen in liquid nitrogen, and stored at −80 °C until assayed. The tissue samples were homogenized in a Potter–Elvehjem glass homogenizer in Tris-HCl buffer (0.1 M, pH 7.4) to 2 % (w/v) homogenate and centrifuged for 10 min at 1 000 g at 4 °C to remove unbroken cells and particulate debris. The supernatants were used for the determination of the activities of succinate dehydrogenase (SDH) (E.C. 1.3.99.1) (Earl and Korner 1965), malate dehydrogenase (MDH) (E.C. 1.1.1.37) (Ochoa 1955), citrate synthase (CS) (E.C. 4.1.3.7) (Srere 1962), phosphofructokinase (PFK) (E.C. 2.7.1.11) (Bourdon *et al.* 1974), and hexokinase (HK) (E.C. 2.7.1.1) (Beutler *et al.* 1969). The activity of lactate dehydrogenase (LDH) (E.C. 1.1.1.27) was determined using commercial reagents (Boehringer Mannheim). The protein concentrations of homogenates was estimated by the phenol method (Lowry et al. 1951). For the determination of glycogen content, the muscle samples were homogenized in 1 M-HCl and heated at 100 °C for 2 h. The glycogen content was then analyzed as glucosyl units according to the method of Huijing (1970). Measurements were made at 37 °C with Cary 118 double-beam spectrophotometer. The assays of the four animal groups were always performed together.

Samples of ISBAT were fixed in 10 per cent formol. After dehydration with alcohol and embedding in paraffin, the 7 µm sections were stained with hematoxylin-eosin. The density of fat cell nuclei was calculated from photomicrographs.

Results

The results in Table I show that repeated thyroxine injections retarded the weight gain of the rats. Furthermore the heart, adrenals and ISBAT were larger in thyroxine-treated rats than in the controls. This increased size of ISBAT was not due to hyperplasia of the tissue but most probably to increased fat content of the tissue.

Histological observation of the tissue revealed that the color of the tissue was pale and the fat droplets inside the fat cells were mostly large and unilocular and the density of fat cell nuclei was decreased (Table I). All these changes were observable also in the animals having

Acta physiol scand, 1978, 103 52–58

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Alprenolol fails to antagonize the metabolic changes following repeated thyroxine injections in the rat

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Received 7 November 1977

Abstract

HARRI, M N E. *Alprenolol fails to antagonize the metabolic changes following repeated thyroxine injections in the rat* Acta physiol scand 1978 *103* 52–58

Repeated injections of rat with l thyroxine (50 μg/kg daily for 5 five-day weeks) retarded the weight gain of the animals and increased the absolute and relative size of the heart, adrenals and interscapular brown adipose tissue In the myocardium and thigh muscle, thyroxine treatment resulted in elevated activity of oxidative enzymes, succinate dehydrogenase, malate dehydrogenase and citrate synthase, while the activities of glycolytic enzymes remained unchanged Glycogen content of the heart was decreased following thyroxine regime. In the brown fat, on the other hand thyroxine injections resulted in a reduction of the activity of oxidative enzymes. This reduction can be accounted for by the decreased protein (enzyme) content of the tissue due to deposition of fat. Furthermore, thyroxine treatment delayed the body cooling of the rats swimming in water at 25°C and enhanced hyperthermic response to injected noradrenaline. All these changes, which were not observable in rats treated with daily alprenolol (20 mg/kg) injections, were as pronounced in rats injected with alprenolol together with thyroxine as in rats injected with thyroxine only It is concluded that beta blockers do not antagonize the metabolic changes due to hyperthyroidism.

Key words: Hyperthyroidism metabolic changes, body cooling, non-shivering thermogenesis, myocardium, skeletal muscle, brown adipose tissue, beta blockers

Although the beta adrenergic blocking agents have been employed with satisfactory results in the treatment of the many clinical manifestations of hyperthyroidism (Riddle and Schwarz 1970 Grossman *et al* 1971 a, b) the mechanism for this action is uncertain. Beta blockers apparently do not influence the thyroid gland or the peripheral metabolism of thyroxine (Azizi *et al* 1974) There are, however newer data according to which beta blockers could influence metabolism of thyroxine (Theilade *et al* 1977) Beta blockers did not abolish the metabolic changes due to experimental hyperthyroidism in the guinea pig (Otter *et al* 1974) However beta blockers reduce the response of body temperature, blood free fatty acids or lactate to exercise in hyperthyroid dogs (Kaciuba Uścilko *et al* 1976) Furthermore reduced beta adrenergic responsiveness has been reported in hypothyroid rats (Fregly *et al* 1977). There are also evidence that prolonged alprenolol treatment could decrease the responsiveness of beta receptors in the rat (Harri 1977).

The present investigation was undertaken in order to elucidate whether or not beta blockers, when given simultaneously with thyroxine, could antagonize the thyroxine induced metabolic changes in heart or skeletal muscle (Kubista *et al.* 1971) or in brown adipose tissue (LeBlanc and Villemaire 1970, Ricquier *et al.* 1976, Rabi and Cassuto 1976). The ability of prolonged beta blockade to reduce the enhanced heat production capacity which results from thyroxine injections, was tested by measuring the heat loss of swimming rats (Dawson *et al.* 1970) or the calorigenic response to injected noradrenaline (LeBlanc and Villemaire 1970).

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TABLE I Effect of daily (for 25–26 days) injections of alprenolol (20 mg/kg), l-thyroxine (50 μg/kg) and their combination on body and organ weight and on relative density of nuclei in histological sections of ISBAT

	Control (18)	Thyroxine (14)	Thyroxine + Alprenolol (15)	Alprenolol (4)
Initial body wt, g	202 ± 5.29	210 ± 7.27	216 ± 6.93	206 ± 2.99
Δ Body wt, g	156 ± 4.14	14[illegible] ± 2.85	135 ± 3.34	145 ± 2.48
Heart, mg	825 ± 20.2	850 ± 28.6	792 ± 15.1	728 ± 15.2*
Heart, mg/100 g	270 ± 4.4	300 ± 9.8*	299 ± 4.7*	250 ± 7.0*
Adrenals, mg	55.1 ± 2.15	67.9 ± 4.47*	73.1 ± 2.61	60.8 ± 2.59
Adrenals, mg/100 g	18.0 ± 0.57	24.1 ± 1.87*	27.6 ± 1.28	20.9 ± 0.84*
ISBAT, mg	377 ± 23.7	482 ± 25.8	466 ± 26.7	237 ± 18.2*
ISBAT, *mg/100 g*	*122 ± 4.9*	*171 ± 10.6*	*177 ± 12.3*	8[illegible] ± 5.7***
Nuclei/mm in ISBAT	2 162 ± 61	1 610 ± 90*	1 585 ± 88*	2 644 ± 155*

ISBAT = interscapular brown adipose tissue.
Number of rats in parentheses. Significant difference from the controls *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Student's t-test).

received alprenolol injections together with thyroxine, in spite of the fact that alprenolol treatment alone decreased the size of the heart and ISBAT and increased the density of fat cell nuclei in the brown fat. The adrenals were enlarged in all drug treated animals.

The results in Table II show the enzymatic changes due to drug treatment. In the myocardium, thyroxine injections increased the activities of oxidative enzymes, MDH and CS. Other enzymes studied did not show any significant changes. Alprenolol injections, when given together with thyroxine, did not abolish these changes: the increase in CS activity was as great as in animals injected with thyroxine only. The increase in the activity of MDH did not attain the level of statistical significance, due to great individual variation. A decrease in the heart muscle glycogen content was seen both in animals treated with thyroxine only and in animals injected with alprenolol in combination with thyroxine. No significant changes were observable in animals having received alprenolol injections only.

The thigh muscle demonstrated increased activity of oxidative enzymes, SDH, MDH and CS in both thyroxine-treated groups. No other changes were found.

In the ISBAT, on the other hand, a decrement in the activity of the oxidative metabolism was noted. The decrement in the activity of SDH was statistically significant in both thyroxine treated groups and that of MDH and CS in the animals treated with thyroxine only, although a similar tendency was observable in animals having received alprenolol injections in combination with thyroxine. The protein content of the tissue was again decreased in both thyroxine-treated animal groups. A tendency of the increased activity of oxidative enzymes and protein content was evident in rats having received alprenolol injections only. Due to small number of rats, however, these changes did not attain the level of statistical significance (*cf.* Harri 1977).

Fig. 1 shows the rate of cooling of rats swimming in water at 25°C. The similarities in the initial slope of the lines indicate that the initial rate of cooling was identical in all groups and corresponds to a cooling constant of 4.13 C/h when calculated according to the formula of $C = 2.3\, d \log (T_B - T_A)/dt$ (Morrison and Tietz 1957), where T_B and T_A refer to body and

TABLE II. Effect of daily (for 25–26 days) injections of alprenolol (20 mg/kg), l-thyroxine (30 µg/kg) and their combination on enzyme activities and protein and glycogen concentrations of rat tissues.

	Control	Thyroxine	Thyroxine + Alprenolol	Alprenolol
Myocardium				
Succinate dehydrogenase	39.1±1.01	39.0±0.80	39.1±1.03	37.3±1.76
Malate dehydrogenase	679±12.5	722±9.53	719±16.3	696±33.9
Citrate synthase	113±1.85	122±2.00**	122±1.87**	112±0.91
Phosphofructokinase	47.8±4.18	41.4±9.34	56.2±6.94	44.4±6.50
Hexokinase	3.43±0.16	3.53±0.21	3.06±0.21	3.02±0.24
Lactate dehydrogenase	891±21.9	931±27.3	894±9.55	872±16.1
Protein	88.6+1.05	92.6±0.40	89.7±1.45	89.1±1.23
Glycogen	4.13+0.33	2.57±0.25**	2.96±0.18**	3.22±0.11
M. vastus lateralis				
Succinate dehydrogenase	6.22±0.22	6.99±0.23*	6.98±0.28*	5.77±0.24
Malate dehydrogenase	188±4.77	200±5.33	214±12.7*	180±14.6
Citrate synthase	28.2±0.50	27.3±0.94	31.2±1.86	26.0±1.19
Phosphofructokinase	16.1±1.23	18.7±2.44	17.6±1.40	18.5±2.56
Hexokinase	3.12±0.21	3.72±0.23	3.81±0.39	3.10±0.57
Lactate dehydrogenase	1 030±24.8	1 086±31.7	1 060±20.7	1 030±47.4
Glycogen	5.20±0.52	4.54±0.52	5.40±0.57	5.19±1.10
ISBAT				
Succinate dehydrogenase	23.4±1.17	19.0±0.84**	17.9±0.72***	23.2±1.76
Malate dehydrogenase	449±24.1	317±16.5**	409±31.7	473±30.5
Citrate synthase	69.3±7.40	49.7±4.79*	54.4±4.82	83.3±8.47
Hexokinase	1.39±0.21	1.16±0.14	1.13±0.17	—
Lactate dehydrogenase	254±27.2	209±19.6	229±41.5	260±10.7
Protein	73.8±3.00	59.5±5.01	57.8±2.70**	79.5±[illegible]85

ISBAT = interscapular brown adipose tissue. The number of rats is identical to those given in Table I. Enzyme activities are expressed as µmoles of substrate utilized per min per g wet weight, and protein and glycogen as mg/g wet tissue weight. Significant differences from the controls: *p < 0.05, **p < 0.01 and ***p < 0.001 (Students' t-test).

ambient temperature, respectively. However, later the rate of cooling slowed down in the thyroxine-treated groups, the time of the T_R to reach 30 C was significantly longer both in the animals treated with thyroxine only and in animals having received alprenolol injections in combination with thyroxine than in the control rats or in the rats treated with alprenolol only.

Fig. 2 shows the metabolic response of the rats to injected noradrenaline (0.5 mg/kg i.p.). In both thyroxine-treated groups noradrenaline induced a significant rise in the colonic temperature, while in other groups it failed to cause hyperthermia.

Discussion

In accordance with earlier findings, the heart and ISBAT were smaller in alprenolol-treated rats than in the controls (Harri 1977). In spite of that, the organ weights in rats treated with alprenolol in addition to thyroxine more closely resembled those of the rats injected with thyroxine only. Enlargement of the adrenals in all drug-treated animals can be regarded as an indication of non-specific stress resulting from the charge of drugs. It is interesting to note that this enlargement was greatest in animals having received both alprenolol and thyroxine injections than in animals subjected to one drug only.

The unchanged activity of tissue enzymes in rats treated with alprenolol only (at a room

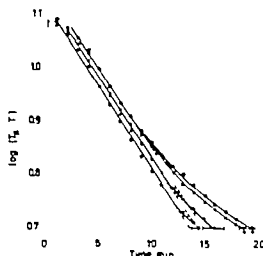


Fig. 1 The rate of body cooling in rats swimming in water at 25 C. ● = control rats, Δ = alprenolol-treated rats (20 mg/kg daily for 5 five-day weeks), O = thyroxine-treated rats (50 μg/kg daily for 5 five-day weeks), and × = rats injected with alprenolol together with thyroxine T_B T_A = the difference of body and ambient (water) temperature. The time of the body temperature to cool to 30°C is indicated at the end of each line as the mean ± S.E. The broken lines extrapolate the initial rates of body cooling = significantly different from the controls (p 0.001 Student's t-test). Five rats per group.

temperature of 20–21 C) is not only due to a small number of rats in this group but is in accordance with an earlier more detailed investigation (Harri 1977)

The enzymatic responses of the heart and skeletal muscles to thyroxine were relatively small and they were seen in the oxidative enzymes only. This is not very surprising because in an earlier study the enzymatic changes even to massive dosage of thyroid hormone did not reach more pronounced values (Kubista *et al* 1971). The increment in the activity of HK which was very marked in the study of Kubista *et al* 1971 was not statistically significant in the present study. The response of HK to a single exercise bout (Barnard and Peter 1969 Boström *et al* 1974) indicates the acute nature of the response. It is thus possible that the response of HK to a few injections of massive doses of thyroxine (Kubista *et al* 1971) is not comparable to the response observed in this study as a result of prolonged treatment with a much smaller dosage.

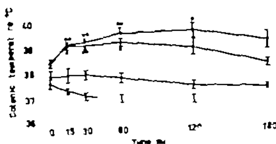
However the most interesting observation of the present study was that alprenolol when given together with thyroxine failed to antagonize thyroxine-induced enzymatic changes.

The significance of the decreased glycogen content in the heart but not in the skeletal muscle in thyroxine-treated rats remains to be clarified by further experiments. The decrease in the glycogen content possibly reflects elevated aerobic carbohydrate catabolism of the myocardium as suggested by Kubista *et al* 1971. This indicates that the response of the heart muscle to thyroid hormone can be different from that of especially white skeletal muscle (Kubista *et al* 1971). From the point of view of the present study however it is interesting to note that the glycogen drop was as great in animals having received alprenolol in combination with thyroxine as in animals having received thyroxine injections only.

The metabolic response of ISBAT to thyroxine regime *i.e* the decreased activity of mainly oxidative enzymes was coupled with a decrease in the concentration of protein in the tissue.

Histological observation revealed that the ISBAT from thyroxine-treated rats was pale in color and the fat droplets were large and mostly unilocular. Accumulation of fat in the ISBAT following hyperthyroidism has been reported earlier in hamster (Rabi and Cassuto 1976) and rat (Ricquier *et al* 1976). It can thus explain the decreased density of fat cell nuclei and the decreased protein concentration observed in the present study in these animals.

Fig. 2. The response of the body temperature to injected noradrenaline (0.5 mg/kg s.c.) at 29°C in control rats (●), in alprenolol-treated rats (20 mg/kg daily for 3 five-day weeks) (△), in thyroxine-treated rats (50 µg/kg daily for 5 five-day weeks) (○), and in rats injected with alprenolol together with thyroxine ( ). Vertical bars indicate ± S.E. Noradrenaline was injected at 0 min. Asterisks mark those hyperthermic changes which significantly differ from the initial value (0 min): *p 0.05, **p 0.01 and ***p 0.001. Four rats per group.



The ISBATs of the rats treated with alprenolol in addition to thyroxine more closely resembled those of the animals treated with thyroxine only than those of animals injected with alprenolol only.

When the difference between body and water temperatures is large in swimming rats, the heat loss by convection greatly exceeds heat production (Dawson *et al.* 1970). In this situation the insulative capacity of the body primarily determines the rate of body cooling. In this study the initial slope of body cooling was very similar in all experimental groups used, an indication that the insulative capacity was about similar in them. Later when the difference between ambient and body temperature becomes smaller the metabolic heat production slows down the rate of body cooling. Thus, the more the rate of body cooling shifts from the initial slope the more metabolic heat production exceeds heat loss.

In this study the rate of body cooling was, in spite of initial identical rate in all groups, significantly slower in the rats treated with thyroxine alone or in combination with alprenolol than in the other groups. Moreover there was neither significant difference between the two thyroxine groups nor between the controls and the rats injected with alprenolol only. Thus a conclusion has to be drawn that alprenolol did not impair the calorigenic effect of thyroxine treatment.

Increased non-shivering thermogenesis, *i.e.* calorigenic response to injected noradrenaline, has been reported to result from prolonged thyroxine treatment (LeBlanc and Villemaire 1970). In this study the increase in colonic temperature after noradrenaline injection was used as a measure of non-shivering thermogenesis. It was observable in rats treated with thyroxine alone as well as in rats having received alprenolol injections in combination with thyroxine but not in other experimental groups. Here again, it can be concluded that alprenolol treatment did not abolish or diminish the enhanced non-shivering thermogenesis due to thyroxine regime.

In conclusion, the present results demonstrate that none of the measured parameters, which are influenced by prolonged thyroxine injections, could be antagonized by alprenolol injections given together with thyroxine. These findings are even more obvious because alprenolol treatment alone either did not induce any changes or caused changes, which were opposite to those of thyroxine. Thus the beneficial effect of beta blockers in the treatment of many clinical manifestations of hyperthyroidism (Riddle and Schwarz 1970, Grossman *et al.* 1971 a, b) cannot be accounted for by metabolic way of effect.

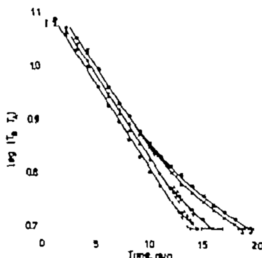


Fig. 1 The rate of body cooling in rats swimming in water at 25°C. ● = control rats, Δ = alprenolol-treated rats (20 mg/kg daily for 5 five-day weeks), O = thyroxine-treated rats (50 μg/kg daily for 5 five-day weeks) and × = rats injected with alprenolol together with thyroxine. T_B–T_A = the difference of body and ambient (water) temperature. The time of the body temperature to cool to 30°C is indicated at the end of each line as the mean ± S.E. The broken lines extrapolate the initial rates of body cooling. = significantly different from the controls (p 0.001 Student t-test). Five rats per group

temperature of 20–21 C) is not only due to a small number of rats in this group but is in accordance with an earlier more detailed investigation (Harri 1977)

The enzymatic responses of the heart and skeletal muscles to thyroxine were relatively small, and they were seen in the oxidative enzymes only This is not very surprising because in an earlier study the enzymatic changes even to massive dosage of thyroid hormone did not reach more pronounced values (Kubista *et al* 1971). The increment in the activity of HK, which was very marked in the study of Kubista *et al* 1971 was not statistically significant in the present study The response of HK to a single exercise bout (Barnard and Peter 1969 Boström *et al* 1974) indicates the acute nature of the response. It is thus possible that the response of HK to a few injections of massive doses of thyroxine (Kubista *et al.* 1971) is not comparable to the response observed in this study as a result of prolonged treatment with a much smaller dosage.

However the most interesting observation of the present study was that alprenolol when given together with thyroxine failed to antagonize thyroxine-induced enzymatic changes.

The significance of the decreased glycogen content in the heart but not in the skeletal muscle in thyroxine treated rats remains to be clarified by further experiments. The decrease in the glycogen content possibly reflects elevated aerobic carbohydrate catabolism of the myocardium as suggested by Kubista *et al* 1971 This indicates that the response of the heart muscle to thyroid hormone can be different from that of especially white skeletal muscle (Kubista *et al* 1971) From the point of view of the present study however it is interesting to note that the glycogen drop was as great in animals having received alprenolol in combination with thyroxine as in animals having received thyroxine injections only

The metabolic response of ISBAT to thyroxine regime, *i.e* the decreased activity of mainly oxidative enzymes was coupled with a decrease in the concentration of protein in the tissue.

Histological observation revealed that the ISBAT from thyroxine treated rats was pale in color and the fat droplets were large and mostly unilocular Accumulation of fat in the ISBAT following hyperthyroidism has been reported earlier in hamster (Rabi and Cassuto 1976) and rat (Ricquier *et al* 1976) It can thus explain the decreased density of fat cell nuclei and the decreased protein concentration observed in the present study in these animals.

Acta physiol. scand. 1978. 103. 59–70

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On the chemical nature of the blood borne cardiotoxic material released from the feline small bowel in regional shock

By

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Received 10 November 1977

Abstract

Lundgren, O. and U. Haglund. *On the chemical nature of the blood borne cardiotoxic material released from the feline small bowel in regional shock.* Acta physiol. scand. 1978. *103.* 59–70.

The cardiotoxic material released into blood from the feline small intestine during a 2 or 3 h regional shock period (inflow pressure to the small intestine 30–35 mmHg during a continuous activation of the regional sympathetic vasoconstrictor fibres at 6 Hz) has been analyzed with regard to three properties: 1. molecular mass determined by molecular filtrations; 2. solubility in a nonpolar solvent (ether); 3. heat stability by heating plasma to 80°C for 30 min. The results obtained suggest that the cardiotoxic material consists of at least two heat stable fractions. One is water soluble with a molecular mass between 500 and 1 000 d and the other is lipid soluble with an unknown molecular mass.

It is well established that ischemia in the splanchnic region may produce a shocklike state in an experimental animal (for a recent review see Marston 1977). The pathogenesis of this shock situation is, according to some authors, secondary to the release of toxic material from the hypoxic tissue (for reviews, see Gruber 1967 and Lefer 1973). In this laboratory we have demonstrated that simulating a shock situation in the feline small bowel by lowering arterial inflow pressure to 30–35 mmHg for 2 or 3 h during a concomitant activation of the regional sympathetic vasoconstrictor fibres at 6 Hz almost invariably produces tissue damage, particularly at the villous tips. The development of these lesions is, at least in part, due to an exaggerated extravascular shunting of oxygen in the intestinal countercurrent exchanger as a result of a reduced linear flow rate in the villous vascular loops (*cf.* Haglund *et al.* 1975, Haglund and Lundgren 1974). After such a simulated intestinal shock period in an otherwise normotensive animal the cardiovascular system deteriorates, as judged from a rapid blood pressure fall of 40–50 mmHg during the first hour after the regional hypotension (Haglund and Lundgren 1973, Haglund *et al.* 1976 b). Perfusing the gut lumen with an oxygenated saline solution prevented the development of the tissue lesions as well as the cardiovascular collapse (Haglund *et al.* 1976 a, b). From these and other studies (Haglund and Lundgren 1973) it was inferred that blood borne

This work was supported by The Finnish Cultural Foundation—North Savo Fund, The Finnish Scientific Society, The Finnish Heart Association, and The Ida Montin Foundation. Valuable technical assistance by Mrs Riitta Venäläinen is greatly appreciated. Alprenolol was a generous gift from AB Hässle, Sweden.

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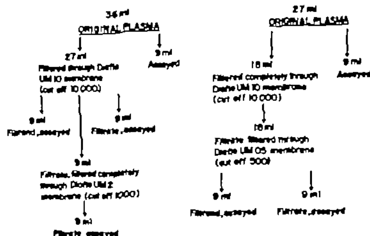


Fig. 1. The two filtration procedures used, schematically illustrated.

To obtain control plasma, identical experiments to those described above were performed except that no regional intestinal shock was induced.

B. *Histological technique*

30–60 min after the exchange transfusion the small intestine was excised and weighed. In most expts. a rectangular piece (about 4 cm) of the mid gut wall was cut out and after mounting it on cork, it was fixed in 10% neutral formalin. The tissue specimen was then prepared with routine histological techniques and stained with hematoxylin and eosin. As described earlier (Chiu et al. 1970, Åhrén and Haglund 1973) the morphological changes in the mucosa were graded into 6 grades, where grade 0 means normal mucosa and 1 to 5 denote increasing damage to the intestinal epithelium. The pathognomonic lesion for grade 1 is the development of subepithelial space at the tip of the villus. This space is more extended in grade 2 where also epithelial lifting is seen. In grade 3 there is massive epithelial lifting also along the sides of the villus and in grade 4 the villus is denuded. Grade 5 is characterized by disintegration of the lamina propria, hemorrhage and ulceration in the mucosa, while the deeper layers of the intestinal wall are intact.

It was demonstrated in control expts (Haglund and Lundgren, unpublished observations) that the tissue damage, as judged by the histological appearance, is the same in all parts of a small bowel subjected to a period of regional shock.

C. *Biochemical procedures*

The different plasma samples were subjected to 3 different procedures, ultrafiltration, ether extraction and heating.

1. *Molecular ultrafiltration.* Control and shock plasma was subjected to two different molecular filtration procedures (Fig. 1), using Diaflo molecular filters (manufactures by Amicon Corp., Lexington, Mass.). The filtrations were carried out in a magnetically stirred ultrafiltration cell (Amicon Model 52) at 20–22°C at a pressure of 344 kPa (50 psi). Before each plasma filtration about 50 ml of a sterile, pyrogen free, isotonic saline solution was filtered through the membrane to be used. Filtration rate was determined and if the magnitude of that rate deviated markedly from earlier filtrations with the same filter or filters of the same type, the filter was discarded.

The details of the two filtration procedures are depicted in Fig. 1. With the procedure shown in the left part of the figure four plasma samples were obtained: original plasma, UM 10 filtrand (containing solutes with a molecular mass above 10 000 d concentrated three times), UM 10 filtrate (solutes with a molecular mass below 10 000 d) and UM 2 filtrate (solutes with a molecular mass below 1 000 d). Similarly in the other filtration procedure illustrated in the right part of Fig. 1, three plasma solutions were obtained: original plasma, UM 05 filtrand (containing solutes with a molecular mass between 500 and 10 000 d concentrated two times) and UM 05 filtrate (containing solutes with a molecular mass below 500 d).

2. *Molecular filtration and ether extraction.* In this series of expts. plasma from control and shocked

material is released from the villous tissue lesions, producing hypotension in the experimental animal

A negative inotropic influence on the heart constitutes one important mechanism for the development of the shock like state described above. This conclusion is based on *in vivo* studies (Haglund and Lundgren 1973 Haglund *et al.* 1978) as well as on *in vitro* studies using two different myocardial preparations (Lundgren *et al.* 1976) In the latter study it was demonstrated that exposing rabbit papillary muscle to cat plasma, collected in the intestinal vein immediately after a simulated shock period, reduced peak isometric tension to half of control value while control plasma had no such effect. Hence, it was concluded that the feline small intestine in shock produced material with a negative inotropic effect on the heart.

The present study represents an attempt to chemically characterize this material as a first step in an eventual chemical isolation and identification Three questions have been considered in this study: What is the molecular mass of the material? Is it soluble in nonpolar solvents? Is it heat stable or heat labile?

Methods

A *Experiments on cats*

The experimental procedures for the studies on cats have been described in detail elsewhere (Haglund and Lundgren 1973) and only a brief summary is given here. The animals had been deprived of food for 12 h and had no obvious signs of intestinal diseases. They were anesthetized with chloralose (50 mg/kg b.wt., i.) A major portion of the small bowel (weight 50–70 g) was isolated and the remainder of the intestine, the spleen, the greater omentum and the major part of the pancreas were extirpated The superior mesenteric artery supplying the isolated intestinal segment, was surrounded by an adjustable clamp so that the regional arterial inflow pressure could be lowered to any desired level. The nerves surrounding the superior mesenteric artery were cut and their distal ends were placed on a ring electrode for electrical stimulation. The left adrenal gland was denervated and the vessels supplying the right adrenal were ligated. Atropine (1 mg/kg b.wt.) was given i.

After heparinization, the left femoral artery and minor branch of the superior mesenteric artery distal to the adjustable clamp were cannulated to record arterial pressure by pressure transducers (Statham P23). The mesenteric vein was cannulated and the venous outflow from the intestinal segment and its lymph nodes was recorded continuously by an optical drop recorder coupled to a ordinate writer Venous outflow pressure was set at 10 mmHg. The blood was returned t the animal through catheter in the jugular vein Recordings were made on a Grass polygraph.

A slow i. infusion of a glucose solution containing bicarbonate (10 mmol of N HCO$_3$ per 100 ml of 10% glucose solution, given at a rate of 0.1–0.2 ml/min) was started on induction of anesthesia and continued throughout the expt. This infusion has been shown to maintai arterial pH at normal level, despite the operative trauma and intestinal hypotension (Haglund and Lundgren 1972). The tubes and the funnel draining the intestinal venous blood were primed with 6 % dextra solution (1/3 low molecular weight dextran (mean molecula mass 40 000 d) and 2/3 medium molecular weight dextran (mean molecular mass 70 000 d) Pharmacia, Uppsala, Sweden) Further 2 3 ml of this solution was given to the animal every 20 min during the hypotensive period

After completion of the operative procedures a period of 30–40 min was allowed t pass. Then an intestinal shock was mimicked by lowering arterial nflow pressure to 30–35 mmHg during a continuous electrical stimulation of the regional vasoconstrictor f bres t 6 Hz with square wave pulses of 6 ms and 12 V These manipulations produced a low flow state in the small intestine simila t that seen in severe hemorrhagic shock (Haglund 1973). It should be pointed out that the remaining part of the pancreas as perfused at normal arterial pressure *via* the celiac artery Immediately after 3-hour period of regional shock, the intestinal venous outflow was collected in a cooled plastic beaker for bout 5 min (80 ml) with a corresponding substitution of fresh blood from another cat. After centrif gation the plasma ("shock plasma") was stored in plastic tubes at − 20 C until tested on the bioassay systems (see Bioassay technique).

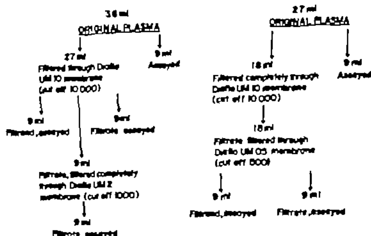


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Statistics

he quantitative effect of any given test solution on the papillary muscle was defined as the ratio (R) of eak isometric tension developed in the test solution (plasma) to that developed in the control bathing nodified Krebs-Henseleit solution, i.e.

$$R = \frac{\text{peak isometric tension, test solution}}{\text{peak isometric tension, control solution}} \quad (1)$$

More than one muscle was used to test each plasma sample, and to calculate the average value the geometric rather than arithmetic mean was used. Thus, if the isometric tension of two muscles doubled and that of two muscles was halved, the geometric mean was 1.00 in contrast to the arithmetic mean of 1.25. The geometric mean isometric tension ratio ($\bar{R}$) of n muscles was calculated conventionally as

$$\bar{R} = \text{antilog} \frac{1}{n} \sum \log R$$

For statistical purposes log $\bar{R}$ was used. Statistical significance was calculated by Wilcoxon's test for two samples (Diem and Lentner 1970) or by the sign test (Siegel 1956). A p-value of 0.06 or less was judged significant. The significance level of 0.06 was chosen since the sign test gives this p-value when 6 of 7 observations show the same trend (Siegel 1956).

Results

A. Experiments on cats

The results of the cat experiments are summarized in Fig. 3. It can be seen that lowering arterial inflow pressure to 30–35 mmHg during a continuous stimulation of the regional sympathetic vasoconstrictor fibres induced an initial pronounced vasoconstriction, intestinal blood flow being reduced to 10–20 % of control. Within 20–40 min blood flow had increased to a steady state level of about 25 % of control, evidently due to a decrease of regional flow resistance. In the control expts. blood pressure and intestinal blood flow remained fairly constant throughout the expts.

The results of the grading performed on the histological sections of most of the experimental animals are given in Table I. The small bowel of control animals was graded as 0 or 1 while the gut from most shock animals exhibited mucosal lesions rated as grade 4 or 5.

B. Experiments on isolated rabbit papillary muscles

1. *Molecular filtration.* The results of the two filtration procedures used are shown in Table II (log $\bar{R}$ values) and III (plasma electrolyte concentrations and time to peak tension). The 4 plasma fractions obtained by filtering through UM 10 and UM 2 filters (left panel of Fig. 1) were assayed on 230 rabbit papillary muscles (control plasma 122, shock plasma 108). Three plasma fractions were produced by the UM 10 and UM 05 filtrations (right panel of Fig. 1) and tested on 114 rabbit papillary muscles (control plasma 47, shock plasma 67).

All shock plasma fractions except the UM 10 filtrate (< 10 000 d) and the UM 05 filtrate (< 500 d) differed significantly from the controls (Table II). No statistically significant difference between control and shock plasma samples was found with regard to their electrolyte concentrations (Table IV). Time to peak tension was only slightly affected by the different plasma samples (Table IV).

When comparing the effects on the papillary muscles of the different control plasma fractions with each other it was found that the UM 10 filtrate (< 10 000 d) differed signifi-

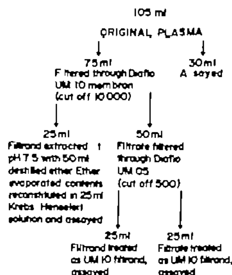


Fig. 2. The filtration procedure employed when performing etherextractions. The original plasma sample consisted of pooled plasma from three cat experiments.

animals were subjected to a filtration procedure depicted in Fig. 2. As illustrated the fractionation procedure produced 4 different plasma fractions: original plasma, UM 10 filtrand (concentrated 3 times), UM05 filtrand (concentrated 2 times) and UM05 filtrate. The pH of the 3 lastmentioned solutions was adjusted to 7.5 and extracted with a double volume of distilled ether. The ether was evaporated under nitrogen at 30°C and the ether-soluble solutes were dissolved in the original volume of a Krebs-Henseleit buffer solution (see below) by ultrasonic agitation.

3. *Heating.* To determine if the cardiotoxic material was heat stable, original plasma was heated in a water bath to 80°C for 30 min. The plasma was then centrifugated (12 000 *g*) for 10 min at 4°C and the supernatant was tested.

The different solutions obtained at the various biochemical manipulations described above were analyzed with regard to their Na, K and Ca concentrations in an Eppendorf flame photometer. The pH of the test samples was adjusted to 7.35–7.40 when bubbled with 4% CO_2 in O_2. In a few plasma samples the concentration of one of the mentioned ions was increased by addition of proper amounts of a concentrated solution of the chloride salt of the electrolyte. The sensitivity of the present bioassay system for changes in [Na], [K] and [Ca] was tested and reported in a previous study (Lundgren *et al.* 1976).

D. *Bioassay technique*

Albino Swedish Land rabbits weighing 1.5–2.5 kg were killed by a blow on the neck. The heart was removed rapidly and the right ventricle was opened while immersed in cold oxygenated incubation solution. The papillary muscles were dissected free together with a small piece of the ventricular wall adjacent to the muscle base. The preparations were mounted vertically in mantled temperature-controlled baths (volume 2.0–2.5 ml) between a fixed hook and a force-displacement transducer (Grass FT 03C). The temperature of the bath was kept at 37°C by a thermostat and the solution was continuously gassed with 4% CO_2 in O_2.

After an equilibration period of 30 min the muscle was stimulated at 1 Hz by application of an electrical field between two platinum electrodes. The pulse duration was set at 1 or 2 ms and the stimulus intensity at 50% above threshold. The changes in tension induced by the isometric contractions were recorded on a Grass polygraph. The preparation was first stimulated for at least 1 h. A length-force curve was then determined for each muscle and the length giving 80–90% of the maximal contractile force was chosen as "resting" length for the rest of the experiment. The muscle was allowed to contract for another 30 min before it was exposed to the solution to be assayed. Only one plasma sample was tested on each muscle. After the expts. the length of the muscle was determined *in situ* with a pair of callipers. The wet weight of the muscle was measured on a Cahn, model G electrobalance.

E. *Solution*

The bathing solution used in the papillary expts. was a modified Krebs-Henseleit solution containing (mmol/l) NaCl 120, KCl 3.3, $NaHCO_3$ 20, KH_2PO_4 1.2, $MgCl_2 \cdot 6H_2O$ 1.2, $CaCl_2$ 2.5, glucose 11.5.

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...quantitative effect of any given test solution on the papillary muscle was defined as the ratio (R) of ...k isometric tension developed in the test solution (plasma) to that developed in the control bathing ...fied Krebs-Henseleit solution, i

$$R = \frac{\text{peak isometric tension, test solution}}{\text{peak isometric tension, control solution}} \qquad (1)$$

...re than one muscle was used to test each plasma sample, and to calculate the average value the ...metric rather than arithmetic mean was used. Thus, if the isometric tension of two muscles doubled ...l that of two muscles was halved, the geometric mean was 1.00 in contrast to the arithmetic mean of ...5. The geometric mean isometric tension ratio ($\bar{R}$) of ... muscles was calculated conventionally as

$$\text{antilog}^{1} \sum \log R_i$$

For statistical purposes log $\bar{R}$ was used. Statistical significance was calculated by Wilcoxon's test for ...e samples (Dunn and Leucher 1970) or by the sign test (Siegel 1956). A p-value of 0.06 or less was judged ...gnificant. The significance level of 0.06 was chosen since the sign test gives this p-value when 6 of 7 ...servations show the same trend (Siegel 1956).

Results

A. Experiments on cats

The results of the cat experiments are summarized in Fig. 3. It can be seen that lowering arterial inflow pressure to 30–35 mmHg during a continuous stimulation of the regional sympathetic vasoconstrictor fibres induced an initial pronounced vasoconstriction, intestinal blood flow being reduced to 10–20% of control. Within 20–40 min blood flow had increased to a steady state level of about 25% of control, evidently due to a decrease of regional flow resistance. In the control expts. blood pressure and intestinal blood flow remained fairly constant throughout the expts.

The results of the grading performed on the histological sections of most of the experimental animals are given in Table I. The small bowel of control animals was graded as 0 or 1 while the gut from most shock animals exhibited mucosal lesions rated as grade 4 or 5.

B. Experiments on isolated rabbit papillary muscles

1. *Molecular filtration.* The results of the two filtration procedures used are shown in Table II (log $\bar{R}$ values) and III (plasma electrolyte concentrations and time to peak tension). The 4 plasma fractions obtained by filtering through UM 10 and UM 2 filters (left panel of Fig. 1) were assayed on 230 rabbit papillary muscles (control plasma 122, shock plasma 108). Three plasma fractions were produced by the UM 10 and UM 05 filtrations (right panel of Fig. 1) and tested on 114 rabbit papillary muscles (control plasma 47, shock plasma 67). All shock plasma fractions except the UM 10 filtrate (< 10 000 d) and the UM 05 filtrate (< 500 d) differed significantly from the controls (Table II). No statistically significant difference between control and shock plasma samples was found with regard to their electrolyte concentrations (Table IV). Time to peak tension was only slightly affected by the different plasma samples (Table IV).

When comparing the effects on the papillary muscles of the different control plasma fractions with each other it was found that the UM 10 filtrate (< 10 000 d) differed signifi-

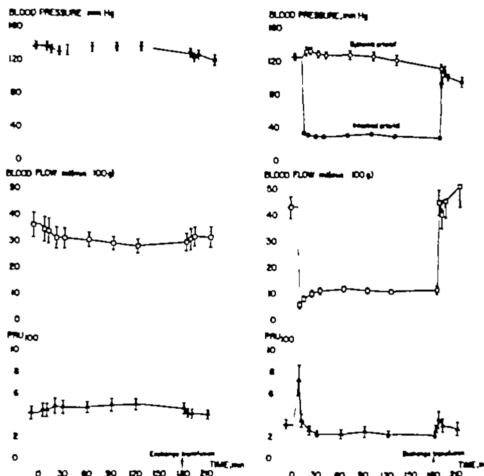


Fig. 3 Changes in arterial blood pressure, intestinal blood flow and intestinal peripheral resistance (PRU_{100}) in control animals (left panel n = 13) and in shock animals (right panel n = 15). Mean values ± SE

cantly from UM05 filtrand (500-10 000 d) and UM2 filtrate (~1 000 d), the log $\bar{R}$ value of the UM10 filtrate being lower than that of the two other fractions. The other control plasma fraction did not otherwise differ from each other or from the original plasma sample.

The striking observation in shock plasma is that the UM05 filtrate (<500 d) was significantly different from all other plasma samples tested (Table IV), the log $\bar{R}$ value of this filtrate being higher than that of all the other fractions (Table II). Furthermore, the UM10 filtrate (<10 000 d) did not depress peak isometric tension as much as the original

TABLE I Microscopic grading of intestinal control segments and of segments subjected to a 3 h period of regional intestinal hypotension. The grading was performed according to Chiu *et al* (1970). Figures denote number of segments

	Grade of mucosal damage					
	0	1		3	4	5
Control	9	3				
Shock			1		7	3

TABLE II The experimental results obtained on rabbit papillary muscles when exposing them to original cat plasma and plasma fractions produced by ultrafiltration through Diaflo UM10, UM2 and UM05 membranes. The effects on peak isometric tension are expressed in terms of the logarithm of the ratio, R, depicting the peak isometric tension developed by the papillary muscle after 15–20 min incubation divided by the peak isometric tension recorded in the control solution. Mean values ± S.E. Numbers within parentheses denote number of plasma samples/total number of muscles assayed. S.E. values were calculated from number of plasma samples. P-values were determined with the nonparametric test of Wilcoxon. ns = not significant.

	Control plasma	Shock plasma	p-value
UM10 and UM2 filtrations			
Original plasma	0.123 ± 0.030 (8/29)	0.279 ± 0.018 (8/33)	<0.01
UM10 filtrand	−0.118 ± 0.047 (7/26)	−0.274 ± 0.041 (7/24)	0.05
UM10 filtrate	0.144 ± 0.018 (8/35)	0.226 ± 0.042 (8/30)	ns
UM2 filtrate	0.112 ± 0.019 (7/32)	−0.231 ± 0.062 (6/21)	0.05
UM10 and UM05 filtrations			
Original plasma	−0.083 ± 0.021 (5/14)	−0.234 ± 0.059 (7/24)	0.05
UM05 filtrand	−0.054 ± 0.025 (5/19)	−0.1 4 ± 0.013 (7/21)	0.05
UM05 filtrate	−0.054 ± 0.068 (5/14)	−0.062 ± 0.023 (7/22)	ns

TABLE III The electrolyte composition of the plasma fractions assayed on the papillary muscles and the time to peak tension measured when exposing the papillary muscles to the Krebs-Henseleit solution and to the different plasma fractions. n denotes the number of plasma samples tested. Time to peak tension was in most cases determined in 4 muscles for each tested plasma sample. The total number of muscles tested are given in Table II. Mean values ± S.E.

	n	Na mmol/l	K mmol/l	Ca mmol/l	Time to peak tension, msec	
					Control	Assay
UM10 and UM2 filtrations						
Original plasma						
Control	8	132.9 ± 3.9	3.71 ± 0.13	2.08 ± 0.07	123.1 ± 2.2	120.9 ± 3.1
Shock	7	137.6 ± 3.0	4.43 ± 0.28	2.08 ± 0.03	120.5 ± 3.9	115.9 ± 5.0
UM10 filtrand						
Control	7	131.0 ± 4.9	3.70 ± 0.21	3.14 ± 0.21	116.6 ± 1.6	115.3 ± 2.6
Shock	6	137.6 ± 4.2	4.13 ± 0.13	2.74 ± 0.12	116.7 ± 2.9	112.7 ± 5.1
UM10 filtrate						
Control	8	139.0 ± 3.5	3.61 ± 0.15	1.12 ± 0.13	117.5 ± 1.6	113.1 ± 2.7
Shock	7	139.5 ± 3.5	4.46 ± 0.37	1.45 ± 0.19	113.1 ± 3.3	113.4 ± 4.0
UM2 filtrate						
Control	7	152.8 ± 13.3	3.42 ± 0.13	1.11 ± 0.13	116.6 ± 2.6	113.1 ± 1.7
Shock	5	141.7 ± 2.9	3.98 ± 0.29	1.21 ± 0.10	121.6 + 3.6	117.7 ± 4.2
UM10 and UM05 filtrations						
Original plasma						
Control	5	143.0 ± 6.9	3.58 ± 0.14	2.14 ± 0.15	128.2 ± 4.7	132.2 ± 7.3
Shock	7	133.2 ± 4.6	4.38 ± 0.46	2.15 ± 0.08	128.3 ± 4.4	128.0 ± 4.4
UM05 filtrand						
Control	5	151.4 ± 5.3	3.80 ± 0.21	2.30 ± 0.17	129.4 ± 4.2	130.0 ± 4.9
Shock	7	153.0 ± 8.2	4.29 ± 0.31	2.01 ± 0.07	131.0 ± 4.2	136.4 ± 8.4
UM05 filtrate						
Control	5	140.2 ± 2.9	4.06 ± 0.14	1.56 ± 0.22	126.4 ± 6.3	127.2 ± 5.1
Shock	7	141.8 ± 9.5	3.88 ± 0.30	1.69 ± 0.22	128.3 ± 3.6	132.9 ± 4.9

TABLE IV p-values when comparing the log R values for the original shock plasma and the different plasma fractions produced by ultrafiltration through Diaflo® UM 10, UM2 and UM05 membranes. The sign test and the Wilcoxon test for two samples were used for the statistical analysis. ns not significant.

	UM 10 filtrand	UM 10 filtrate	UM05 filtrand	UM filtrate	UM05 filtrate
Original plasma	ns	0.06	ns	ns	<0.0_
UM 10 filtrand		ns	<0.01	ns	<0.01
UM 10 filtrate			ns	ns	0.01
UM05 filtrand				ns	0.06
UM filtrate					0.01

shock plasma although the numerical difference between the mean values was fairly small (Table II). The UM 10 filtrand (>10 000 d) exhibited a log $\bar{R}$ value which was significantly lower than that of the UM05 filtrand (500–10 000 d).

2. *Ultrafiltration and ether extraction.* In order to test if the ether extraction as such produced any cardiotoxic material the modified Krebs-Henseleit solution used in this study was extracted with ether as described in Methods. The extracted solutes were reconstituted to the original volume in the Krebs Henseleit solution and assayed. Log $\bar{R}$ measured -0.066 ± 0.08 (mean ± S.E., n 9) in these studies, revealing only a small effect of the ether extraction procedure.

To reduce the number of muscle assays this part of the investigation was performed in the following way: plasma from 3 expts. were pooled. This solution was then filtered as described in Methods (Fig. 2) and tested on papillary muscles. Each muscle assay could in this way be considered to be statistically one observation.

The filtration procedure used in this part of the investigation produced three plasma fractions: UM 10 filtrand (>10 000 d), UM05 filtrand (500–10 000 d) and UM05 filtrate

TABLE V The experimental results obtained on rabbit papillary muscles when exposing them to normal and heated cat plasma and ether-extracted plasma fractions produced by ultrafiltration through Diaflo UM 10 and UM05 membranes. The effects on peak isometric tension are expressed as described in Table III. Mean values ± S.E. Numbers within parentheses denote number of muscles tested (ether extractions), number of plasma samples/total number of muscles tested (heating). p-values were determined with the nonparametric test of Wilcoxon. ns not significant.

	Control plasma	Shock plasma	p-value
Ether extraction			
Original plasma	−0.085 ± 0.051 (7)	−0.234 ± 0.030 (9)	0.0_
Ether-extracted fraction of UM 10 filtrand	−0.131 ± 0.027 (8)	−0.254 ± 0.0_8 (8)	<0.01
Ether-extracted fraction of UM05 filtrand	0.066 ± 0.037 (7)	0.003 ± 0.033 (9)	ns
Ether-extracted fraction of UM05 filtrate	−0.079 ± 0.022 (8)	−0.101 ± 0.011 (6)	ns
Heating			
Original plasma	−0.089 ± 0.019 (6/19)	−0.208 ± 0.038 (8/30)	<0.0_
Heated plasma	−0.104 ± 0.020 (6/18)	−0.[illegible]15 ± 0.016 (8/25)	0.05

...LE VI The electrolyte composition of original plasma and ether-extracted plasma fractions assayed on the papillary muscles and the time to peak tension measured when exposing the papillary muscles to the Krebs-Henseleit solution and the different plasma fractions. Numbers of muscles used for estimating time to peak tension are given in Table V. Mean values ± S.E. – number of plasma samples.

	Na mmol/l	K mmol/l	Ca mmol/l	Time to peak tension, msec	
				Control	Assay
...her extraction					
...riginal plasma					
Control	145	3.2	1.9	127.0±9.5	124.6±10.2
Shock	136	3.4	1.8	121.9±3.9	122.0±4.6
...M 10 filtrand					
Control	148	4.1	1.8	129.3±3.5	126.4±4.3
Shock	146	4.1	2.4	110.1±4.7	106.4±2.6
...M 05 filtrand					
Control	150	4.3	2.2	118.9±8.6	114.3±6.9
Shock	140	4.0	2.6	117.1±[illegible]	112.6±4.2
...M 05 filtrate					
Control	147	4.3	2.2	121.9±4.5	118.1±4.2
Shock	138	4.1	2.5	120.5±3.1	11[illegible].0±3.8
...eating					
...ontrol plasma					
Original plasma (n 6)	140 ±6.3	3.37±0.24	1.97±0.08	129.8±4.2	133.2±6.1
Heated plasma (n 6)	139.3±4.0	3.80±0.23	2.02±0.14	126.2±4.0	121.5±4.6
...hock plasma					
Original plasma (n 7)	134.3±6.6	4.47±0.41	2.12±0.07	126.9±3.8	125.0±4.6
Heated plasma (n 7)	135.0±7.8	4.59±0.34	1.74±0.15	118.4±3.4	118.7±4.6

500 d) (Fig. 2). The assay results obtained when exposing the myocardial tissue to the ther extractable material from these fractions in control and shock plasma are shown in Table V. The results from the assay on the original plasma (not ether extracted) are also hown. Comparing the control and the shock plasma samples revealed a statistically significant difference between the original plasma samples and the UM 10 filtrands (10 000 d, Table V). In both cases the shock plasma was found to cause a more pronounced depression of peak isometric tension of the papillary muscles. Comparing the different control plasma ractions with each other did not unravel any significant differences. However in shock plasma, the original plasma and the UM 10 filtrand was significantly different from the ther extracted parts of the two other plasma fractions. These fractions produced less an effect on the cardiac muscle than the original plasma and the UM 10 filtrand (Table V). The electrolyte concentrations of corresponding control and shock plasma fractions (Table VI) did not differ significantly from each other. Time to peak tension was only slightly affected by the plasma samples (Table VI).

3. *Heating*. 6 control plasma and 8 shock plasma samples were heated to 80°C for 30 min. The effect of this procedure on the papillary peak isometric tension is evident from Table V. With regard to this parameter no effect of heating was observed in either control or shock plasma. Table VI presents the electrolyte concentrations and the papillary time to peak tension of the original and the heated plasma samples. No significant difference between control and shock plasma could be demonstrated with regard to these parameters.

Discussion

The molecular filtration procedures employed revealed at least 2 heat stable substances in intestinal "shock plasma" with a negative inotropic effect on myocardial tissue. One substance has apparently a molecular mass of 500–1 000 d and is insoluble in organic solvents, since control and shock plasma fractions containing molecules with a mass above 500 d differed significantly (Table II), while no difference was found between the ether extracted UM05 filtrands (500–10 000 d Table V). Furthermore, among the different shock plasma fractions (Table IV) the UM05 filtrate with molecules with a mass below 500 d exhibited a significantly higher log $\overline{R}$ value than all other plasma fractions, although the [Ca] of the UM05 filtrate was low (Table III Lundgren *et al* 1976). No such difference in log $\overline{R}$ could be demonstrated for control plasma.

One observation in Table II is, however not consistent with the conclusion above, since log $\overline{R}$ values of the UM10 filtrate (< 10 000 d) of control and shock plasma did not differ significantly Numerically however the UM10 filtrate of shock plasma produced a more pronounced negative inotropic effect on the papillary muscles than control plasma. Furthermore, two other shock plasma fractions containing molecules with a mass below 10 000 d (UM2 filtrate and UM05 filtrand) depressed myocardial contractility more than the corresponding control plasma fractions. Moreover the control UM10 filtrate differed significantly from two other control plasma fractions (UM05 filtrand and UM2 filtrate) suggesting that the filtration procedure as such may have produced an effect on this particular plasma fraction.

The results reported in Table V clearly suggest that shock plasma also contained a cardiotoxic material which was possible to extract by ether at a pH of 7.5 from the high molecular fraction in the UM10 filtrand (> 10 000 d) It seems impossible to estimate the molecular mass of this compound from the molecular filtration expts., since the big proteins in the UM10 filtrand are transport molecules for a number of lipid-soluble solutes of varying molecular mass. The existence of this cardiotoxic material in the UM10 filtrand probably explains the occasional significant difference found in the shock plasma with regard to other plasma fractions than the UM05 filtrate (Table IV).

When exposing the intestine to a period of low arterial blood pressure, it is striking that no significant blood pressure fall occurs during the intestinal arterial hypotension, while a pronounced pressure fall is seen after the regional hypotension. This observation led Haglund and Lundgren (1974) to propose that part of the cardiotoxic material produced in the hypoperfused gut was fairly lipophilic and of comparatively small molecular mass so as to be easily trapped in the intestinal countercurrent exchanger during the period of hypoperfusion, which in turn, delays its net blood absorption The presence of a lipophilic cardiotoxic material was further strengthened by the observations of the present study discussed above.

To summarize, the present study suggests that the feline small intestine in hypotensio produces at least two heat stable compounds with negative inotropic effects on the myo cardium. One is water soluble with a molecular mass of 500–1 000 d and another lipophilic, possibly also of small molecular mass.

The presence of toxic factors in various types of shock has been proposed by a number of estigators as reviewed by Gruber (1967) and Lefer (1973). In the latter review eight fferent "factors" were listed, all of which seem to be formed in the splanchnic region. Most of these are of large molecular mass (above 10 000 d) and the only heat stable water luble "factor" of small molecular mass, with cardiotoxic effects resembling the one of e present study is the myocardial depressant factor (MDF) described by Lefer (1970, 1974). MDF is believed to be a peptide or a glucopeptide having a molecular mass of 800–1 000 d. t is heat stable and soluble in water but not in the nonpolar solvent methylene chloride. In all these respects MDF resembles the hydrophilic cardiotoxic material of the present study. A number of experimental observations suggest, however, that the two cardiotoxic materials may not be chemically identical.

First, MDF is according to Lefer and co-workers mainly released from the ischemic pancreas. In the present shock model a large portion of the pancreas was extirpated and the remaining part was perfused at normal arterial pressure. Second, Glenn and Lefer (1971) were unable to demonstrate the production of MDF by the duodenum *in vitro* although MDF was produced under similar conditions in pancreatic tissue. Third, the myocardial depressant effect of the present study was caused by material in the intestinal venous blood while MDF is transported from the pancreas *via* the lymphatics. Evidence for this was presented by Glenn and Lefer (1970) who showed that thoracic lymph diversion prevented the appearance of MDF in plasma in cats subjected to hemorrhagic shock. Fourth, Lefer and Martin (1969) filtered dialyzed shock plasma through a UM2 membrane (cut off 1 000 d) and found depressant activity in the filtrate but none in the filtrand. In the present study cardiotoxic material was found in plasma fractions containing solutes with a molecular mass below as well as above 1 000 d (Table II). In fact, if MDF is a molecule with a mass below 1 000 d it should have appeared both in the filtrand and in the filtrate of the UM2 membrane. Thus, although the chemical properties of MDF and the water soluble cardiotoxic material of this study exhibit similarities, they may not be chemically identical.

In the review by Lefer (1973), one of the enumerated "factors" is soluble in nonpolar solvents, *i.e.* the reticuloendothelial-depressant substance (RES) described by Blattberg and Levy (1962). This material was soluble in methylene chloride and it also exhibited some cardiotoxic activity (Lefer and Blattberg 1968). However, RES was possible to extract with methylene chloride from a protein free solution (Blattberg and Levy (1966)), while the lipophilic material of this study was found only in the transport protein fraction of plasma.

Fine (1967) has proposed that bacterial endotoxins absorbed from the intestine during shock are one important contributing factor to the development of so called irreversible shock. No determinations of endotoxins were performed in this study. However, endotoxins have been shown not to depress myocardial contractility (Hinshaw *et al.* 1973). Furthermore, endotoxins are water soluble large molecules with mass between 200 000 and 000 000 d. Hence, it seems highly improbable that they are chemically identical to the cardiotoxic material reported in this study.

This study was sponsored by grants from the Swedish Medical Research Council (14X 2855 and 14X-4311), from Magnus Bergvalls Stiftelse, from Fylgias Ärnoldsfond, from Harald and Greta Jeanssons Fond and from the Swedish Society for Medical Sciences.

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Influence of local noxious heat stimulation on sensory nerve activity in the feline dental pulp

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Received 23 November 1977

Abstract

AHLBERG, K. F. *Influence of local noxious heat stimulation on sensory nerve activity in the feline dental pulp.* Acta physiol. scand. 1978. *103* 71–80.

The present investigation was undertaken to develop an experimental model in which noxious heat stimulation was used to produce increased intradental sensory nerve activity in canine teeth of anaesthetized cats. Two techniques were evaluated in which both the method of recording and the nature of the stimulus varied. Slow heating (approx 1°C/s) to 47°C of the tooth surface (combined with recording from electrodes in open dentinal cavities) did not produce any persistent nerve activity. Repeated periods of brief intense heating (approx 60°C/s) (combined with recording from amalgam electrodes placed on cavity floors) resulted in an immediate response and an afterdischarge (phase 3) generally persisting for 20–60 min. Maximum phase 3 activity was characteristic for the individual cat and ranged from 0.2 to 50.2 imp/s, mean value 10.6 imp (S.D. ± 9.2). A systematically higher phase 3 activity was recorded in lower compared to upper canine teeth (p < 0.05). The successive phase 3 response generally occurred after 3–8 stimulations, the median number of required stimuli was 3. Repeated brief heat stimulations combined with the closed cavity recording technique may be used as an experimental model by which the mechanisms behind increases in intradental sensory nerve activity associated with tissue damage can be studied.

Injury to a tooth produced by thermal, chemical bacterial or mechanical insults can result in the clinically observed phenomenon of acute pulpitis with hypersensitivity to stimuli and/or severe pain. Little is known of the mechanisms by which these effects are produced. The purpose of the present study was to develop an experimental model in which thermal stimuli applied locally to the teeth of anesthetized cats could be used to produce similar effects, the degree of which could be assessed by recording the associated intradental sensory nerve activity. Such a model could be used to study the mechanism of acute pulp injury and the effect of interventions which may modify its expression.

Material and methods

The experiments were carried out on adult cats (2–4 kg). The animals were anesthetized with sodium pentobarbital (30 mg/kg i.p.) or chloralose (40 mg/kg) with urethane (50 mg/kg). Immobilization, cavity preparation and experimental procedures were performed as described by Olgart, Haegerstam and Edwall (1974). Two techniques were evaluated in which both the method of recording and the nature of the thermal stimulus were varied.

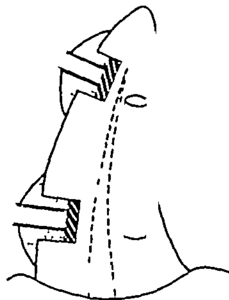


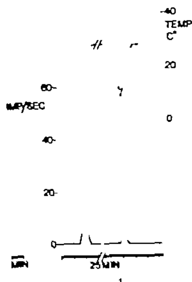
Fig. 1 Drawing showing electrode contacts implanted intradentinally and the tube for the thermistor fixed to the tooth surface.

Open cavity recording—slow heating of tooth

Two cavities were prepared in the canine tooth, one over the pulp horn, the other within the gingival part of the crown. The cavities were filled with isotonic saline solution and surrounded with Plast base (Squibb). Recording electrodes were placed in the cavities and recordings were obtained from intradental nerves (for details see Olgart 1974). The responsiveness of the preparation was tested by the application of 9 sodium chloride solution to the coronal cavity. If no clearcut neural activity was recorded within after applying the hypertonic solution to the exposed dentin, the cavity was deepened until such reactivity was obtained. Washing the cavity with isotonic saline solution rapidly abolished the activity. Tooth temperature, monitored by a thermistor placed at the lingual surface was maintained during control periods at 34°C by a water circulated thermode placed in contact with the tooth crown. Cold stimulation was produced by decreasing the thermode temperature to 20-27°C. The thermal injury in this series of expts was attempted by raising the thermode temperature by approximately 1°C/s to a maximum of 44-47°C. Stimulations were generally applied for 10 min. After stimulation the temperature at the tooth surface returned to resting level at a rate of about 1 C/s.

Closed cavity recording—brief intense heating of tooth

A modified technique developed by Edwall and Olgart (1977) was used to record intradental neural activity (Fig. 1). Two cavities were prepared and deepened to about half the distance from enamel to pulp. After partial drying, the floor of each cavity was covered with a layer of amalgam to which stainless steel tubes (Ø 0.3 mm, length about 2 mm) were secured by composite filling material (Concise). Connecting leads were inserted into the tubes and the signals obtained were amplified and displayed on a cathode ray tube using conventional techniques and stored on magnetic tape (Edwall and Scott 1971; Olgart *et al.* 1974). The signals were counted on a scaler using pulse amplitude discrimination and mean impulse frequency, expressed as imp/s, was printed out for successive 10 s periods. The form and amplitude of recorded spikes did not seem to alter during an experimental session using either recording techniques. Another tube (Ø 1.2 mm length about 4 mm) containing a thermistor was fixed to the tooth surface to register temperature at that site. Responsiveness of this preparation was tested by a cold stimulus produced by application of a cotton pellet soaked with ethyl chloride to the incisal part of the tooth surface for 5–10 s. This caused the surface temperature of the tooth, measured by the thermistor at half the crown length (resting temperature about 30°C), to decrease by 6–10°C. Thermal injury was produced by the application to the tip of the tooth for up to 1 s of guttapercha (De Trey's Provisorische Guttapercha) heated in a flame. This increased the temperature at the surface thermistor to 37–38°C. Experiments in which a thermistor was placed at the tip of the tooth surface, revealed that at the site of application of the guttapercha, the temperature rose to 85–90°C in less than 1 s. Immediately after stimulation the temperature decreased and returned to resting level in 4–5 s. In 3 expts. in which pulp temperature was me... ther



g 2 Influence of cold stimulation on impulse frequency -at, adult 3.0 kg. Pentobarbital. Closed cavity recording. temperature (---) Impulse frequency (—). 1 Cold stimula- on, ethyl chloride

educed into the pulp horn, it rose to maximum of 44–48°C within 2 and returned to resting level in bout 15 after removal of the guttapercha. The corresponding increase in temperature in the middle part of the crown pulp was 7°C (resting pulp temperature 32.5).

T evaluate the longterm effect of repeated brief heat stimulation on the neural activity and on the morphology of the pulp, series of chronic expts as performed (6 teeth, 3 cats). After the first expt, which neural responses were induced by repeated application (5–10 times) of the heat stimulus, the cat was allowed to recover from the anaesthetic. The procedure was repeated 3–4 times at weekly intervals and following the last experimental session the teeth, which had been heated, were extracted and the pulp was examined by conventional histological techniques. Two teeth, prepared in the same way but exposed only to repeated cold stimulation served as controls for the histological studies.

Results

Open cavity recording

6 expts were conducted in 5 cats to study this model. In general no spontaneous activity was recorded, and slow cooling produced only occasional single impulses even on repeated stimulation. Heat stimulation for about 10 min induced a few single impulses or a transient low frequency activity starting when the surface temperature of the tooth had reached 46 C. No activity persisted after stimulation. Repeated and prolonged stimulation (max 30 min) did not provoke any increase in the activity but resulted in a loss of responsiveness to hypertonic saline solution (3 teeth). In these cases exploratory pulp exposure did not produce bleeding which suggested that pulp necrosis had occurred. A thermistor inserted into the pulp revealed pulp temperature of 46°C when the surface temperature of the tooth had been kept at 47°C for 12 min.

Closed cavity recording

No initial spontaneous activity was recorded with the exception of some single impulses immediately after preparation. The cold stimulus resulted in burst of recorded electrical

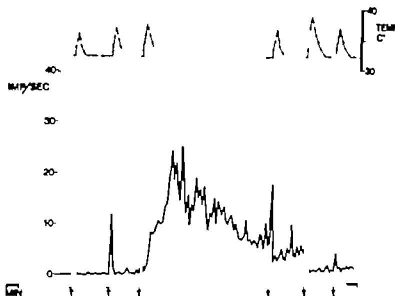


Fig. 3 Influence of repeated heat stimulation on impulse frequency. Temperature (-). Impulse frequency (—). Cat, adult 2.1 kg. Chloralose-urethane. Closed cavity recording. ↑ Heat stimulation, temp. guttapercha.

activity which was closely related to the fall in temperature at the tooth surface and was reproducible (Fig. 2). Brief application of heated guttapercha to the tip of the tooth elicited a burst of impulses generally lasting about 10 s. Initially there was a prompt fall of electrical activity to baseline level, but if the stimulus was repeated this prompt response was succeeded by a longer lasting increase in neural activity which reached a maximum 2–4 min after the stimulus, but was detectable for 20–60 min. This afterdischarge generally appeared after the 2nd stimulus and increased with further stimuli the largest effect being observed after the 3rd to 8th application in different expts. (Fig. 3). Continued application of heat stimuli led to diminution and finally loss of all responses including that associated with cold stimulation

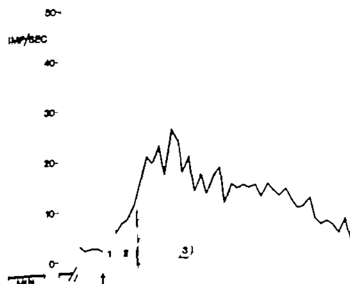


Fig. 4. Impulse frequency response induced by repeated heat stimulation. Closed cavity recording. Arrow indicates third heat stimulation. Phase 1 2 10 s. 2. Phase 2, 3 10 s. 3. Phase 3 18 10 s

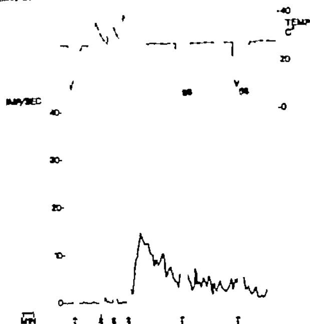


Fig. 5 Influence of thermal stimulation on impulse frequency. Temperature ( ) Impulse frequency (—). Cat, adult 3.0 kg, pentobarbital. Closed cavity recording. 1 Cold stimulation, ethyl chloride. 2 Heat stimulation temporary guttapercha

tion. The prompt response to heat and the more prolonged activity were often separated by a short period when recorded activity approached or reached the baseline level. The complete response can therefore be divided for descriptive purpose into 3 phases, an immediate response (phase 1), a variable latent period (phase 2) and a prolonged afterdischarge (phase 3)

TABLE I. Maximum phase 3 activity in impulses/second, induced by repeated heat stimulation. C+ denotes the right upper and C the left lower canine

Animal no	Maximum phase 3 activity imp/s C	C	C	C	Mean imp/s
1	11.1		16.8		14
2		6.9		8.4	7.7
3		6.9		3.5	4.9
4	2		2.0		2.1
5	1.1	0.9	0.9	1.2	1.0
6		7.8	8.5		8.[illegible]
7	17.6			20.8	19.2
8	1.4	2.7	2.3	3.2	2.4
9	4.9			5.5	5
10	7.0		9.7		8.4
11	35.2			50.2	42.7
12	9.5			15.1	12.3
13	2.1			0.6	1.4
14	7.3			15.7	11.5
15		8.1	14.4		11.3
16		24.6		28.5	26.6

	C C	C− −C	Total
Number of tooth	18	18	36
Means of maximum phase 3 activities	8.7	11.5	10.0



Fig. 6. Section of tooth which has been stimulated with heat on 4 experimental occasions. Note the absence of odontoblastema and the extensiv tissue alterations in the incisal part of the pulp. The [illegible] cavity is seen in the upper right corner

(Fig. 4) A quantitative expression of the phase 3 activity was derived by calculating the mean number of impulses per second over the period from 1–4 min inclusive after stimulation *i.e* excluding the first and second phases of the response If the cold stimulus was applied during a period of phase 3 activity induced by heat, a markedly increased response was obtained compared to control responses (Fig. 5)

The maximum phase 3 activity recorded from 48 teeth in 27 cats and expressed by the above mentioned parameter showed considerable variation between the individual animals and ranged from 0 2 to 50.2 imp/s with a mean value of 10.6 imp/s (S D ± 9 2). The median number of required stimuli was 3 Far less variation in maximum phase 3 activity was



Fig. 7 Same pulp, higher magnification. Demarcation zone showing tissue undergoing necrosis.

and within the individual animals. Table I shows the maximum phase 3 activities obtained in 16 of the cats, in which heat stimulations were applied to one upper and one lower canine tooth or to all four canine teeth. A systematically higher phase 3 activity was recorded lower compared to upper canine teeth ($p < 0.05$ sign test).

In chronic expts. in which animals were studied at weekly intervals, a gradual diminution of the responses was observed and on the 3rd and 4th experimental sessions little or no phase activity could be recorded. The responses to cold and phase 1 response to heat were considerably reduced but still present. Histological examination of these teeth showed severe morphological alterations in the incisal part of the pulp: the odontoblastema was markedly reduced or absent, the structure of the pulp tissue was changed and the number of cells was reduced. In one tooth also a demarcation zone, showing tissue undergoing necrosis was seen in the incisal part of the pulp (Fig. 6 and 7). In control teeth to which only cold stimulation was applied the responses to the stimulus derived at the 3rd or 4th experimental occasion were of about the same magnitude as recorded in the initial expt. These teeth showed only minor morphological changes in the pulp tissue.

Discussion

Thermal stimulation of teeth in cats and dogs has previously been shown to evoke activity in dental nerves (Pfaffmann 1939, Wagers and Smith 1960, Funakoshi and Zotterman 1963, Yamada, Suzuta and Higuchi 1968, Matthews 1967, 1968, 1977), but little information is available about sensitization of intradental nerves following heat stimulation.

Heat stimulations have been found to result in sensitization of nociceptors in skin of cat and monkey (Beck, Handwerker and Zimmermann 1974, Beitel and Dubner 1976, Croze, Duclaux and Kenshalo 1976) and in skeletal muscle of dog (Kumazawa and Mizumura 1977), characterized by a decrease in threshold temperature and an increased frequency of discharge during stimulation. A low-frequency afterdischarge lasting for some minutes was reported by Beitel and Dubner (1976) following long duration of the stimulus (30 s) or large temperature shifts into the noxious heat range. They also found that intense noxious heat depressed the response, which they ascribed to heat damage of the receptor. An after discharge was frequently observed especially on repeatedly heated cases (Kumazawa and Mizumura 1977).

In the present study, brief application of heat to the tooth combined with a closed cavity recording technique was found to be an effective stimulus to obtain a persisting intradental nerve activity. Recent studies have shown that the nervous activity recorded by the present techniques originates from intradental sensory nerves (Arwill *et al.* 1973, Haegerstam 1976) and that it correlates with the sensation of pain in man (Edwall and Olgart 1977). Brief heat stimulation resulted in a complex pattern of neural activity in which 3 phases can be distinguished. The immediate response (phase 1) was probably due to thermal gradients inducing hydrodynamic excitation of the sensory nerves (Brännström and Åström 1972). Phase might be interpreted as a period of latency in which the temperature returns to normal and pathological alterations develop in the injured pulp. The activity during phase 3 is more interesting since it occurred after the stimulus had been removed. This suggests

that phase 3 activity must be due to some persistent damage to the pulp. Support [illegible] interpretation is obtained from a study on monkey by Zach and Cohen (1966). They [illegible] that heat stimulation for 5–20 s, inducing an increase in pulp temperature of about [illegible] resulted in morphological changes such as destruction of the odontoblasts, cell [illegible] hyperemia, edema and, in 15 % of the teeth, pulp necrosis. After an increase of about [illegible] abscess formation was seen in 60 % of the teeth. In the present study a temperature of [illegible] was recorded at the pulp horn, indicating that localized inflammatory changes in the p[illegible] tissue could be expected and evidence of tissue damage was obtained from the histolog[illegible] examination of pulps which had been subjected to repeated heat stimulation in chron[illegible] expts. The finding that repeated heat stimulation was required to produce maximum phase 3 activity indicates that the effects were additive, gradually increasing the severity of the [illegible] damage. This suggestion is supported by the observation that further heat stimulation le[illegible] to diminution of the neural responses in acute as well as in chronic expts. Depressed respons[illegible] following repeated noxious heat stimulation has previously been observed in pulpal nerv[illegible] (Pfaffmann 1939, Wagers and Smith 1960).

The maximum phase 3 activity was found to be characteristic of the individual cat and in [illegible] particular cat the lower canine teeth generally gave a higher activity than the upper canin[illegible] *A quantitative expression of maximum phase 3 activity seems to offer a useful parameter* fo[illegible] comparative studies on intradental sensory nerve activity, especially in homologous pairs of teeth. The variability in maximum phase 3 activity seen within such pairs is not surprising *since the number of activated sensory units available for recording may* differ from on[illegible] preparation to another and the heat stimulations were only roughly standardized. If th[illegible] parameter should be used for experimental evaluation of the sensation of pain as a neural implication of pulp injury it has to be regarded that the present recording techniques only permit recording of peripheral neural activity within the coronal part of the pulp and fro[illegible] only some types of nerve fibres (Haegerstam 1976).

Slow heat stimulation of the tooth in combination with the open cavity recording technique was found to be an unsatisfactory model for studies on increased intradental nerve acti[illegible] despite evident damage to the pulp, indicated by complete loss of reactivity and necros[illegible] It seems unlikely that the difference in recording technique (closed or open cavities) could explain the failure to evoke neural activity by slow heat stimulation, since Edwall and Olgart (1977) reported similar responses using either technique. Another possibility is that the technique to apply the heat stimulus can account for the different results.

Brännström and Åström (1972) proposed that pain elicited by thermal stimuli is produced by fluid movements produced thereby in the dentinal tubules. The poor responses to heat stimulation in the open cavity experiments suggests that raising the temperature at a rate of 1 C/s is inadequate to provoke such hydrodynamic excitation of the intradental sensory nerves. Furthermore, repeated and prolonged heat stimulation caused the loss of responsiveness and impairment of blood supply, suggesting that considerable tissue injury had taken place. One explanation could be that the uniform heating of the pulp produced by this method leads to widespread *functional impairment of tissues including nerves so that the* pulp tissue located between the electrodes from which the recorded electrical activity originates (Haegerstam 1976) is incapable of generating observable impulses. The pulp has been

by Pohto and Scheinin (1958) to be extremely vulnerable to heating, a period of 2 min
46°C being sufficient to lead to arrest of blood flow with stasis and thrombosis ensuing.
… changes could be expected to reduce neural reactivity (Edwall and Scott 1971) and
…ld be occurring at temperatures close to the expected threshold for impulse generation
…nakoshi and Zotterman 1963). Therefore it cannot be excluded that nerve endings
…ned further apically might have been excited though recordings cannot be obtained by
… present techniques. Support for this view can be found in studies where noxious heat
…mulation was applied to teeth and neural activity could be recorded from pulpal fibres in
… alveolar nerv (Pfaffmann 1939, Wagers and Smith 1960, Funakoshi and Zotterman
…3, Matthews 1967, 1968, 1977).
The increased neural activity and excitability produced in the tooth in this study may
…ll be associated with the formation of inflammatory mediators which are known to affect
…ves. If this proves to be the case, the parameters discussed above could be used to evaluate
…g or other influences modifying the inflammatory process. Repeated brief heat stimulation
… combination with the closed cavity recording technique has already been used to examine
… action of antiinflammatory drugs on the responses of pulp tissue to thermal injury
…hlberg 1978).

… investigation was supported by grants from the Swedish Medical Research Council (proj. no. B75-
…X-816-10 and B76-24X-816-11).

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Acta physiol. scand. 1978. 103. 81–91
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Reinnervation of partially denervated rat soleus muscle

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Received 25 November 1977

Abstract

Thompson, W *Reinnervation of partially denervated rat soleus muscle*. Acta physiol. scand. 1978 *103* 81 91

The reinnervation of partially denervated rat soleus muscles by their interrupted, regenerating motor axons has been examined in adult rats. If reinnervation occurred after the remaining intact motor axons had sprouted to their full, maximal extent, then the regenerating axons formed synapses preferentially with denervated muscle fibers and not with fibers innervated by sprouts. The sprouted motor units retained their size as if no reinnervation had occurred. On the other hand, if reinnervation occurred early during the sprouting process, the sprouting motor units were never able to attain their maximal size. Further some muscle fibers became innervated by both sprouted and regenerating axons. These "hyperinnervated" fibers lost their dual innervation within a few weeks. The sprouted axons seemed to be the nerve fibers preferentially eliminated from these hyperinnervated fibers, since during the loss of hyperinnervation the sprouted motor units decreased in size while the motor units formed by the regenerating axons did not change in size. It is proposed that the occurrence of hyperinnervation is influenced by the amount of time sprouting axons have to consolidate their synapses with muscle fibers. Further it is proposed that on muscle fibers which can become hyperinnervated, the sprouted motor neurons are at a disadvantage in the competition for maintenance because of their larger unit sizes.

The number of synapses which a motor neuron forms within its target muscle is limited. In the soleus muscle of the adult rat this limitation is set by a constant or nearly constant number of muscle fibers, a narrowly defined number of motor neurons, and a requirement that each muscle fiber receive innervation from only one neuron. A result of these limitations and an incompletely understood post-natal development (Redfern 1970, Brown *et al.* 1976) is that the soleus motor neurons divide the soleus muscle fibers among themselves so that each neuron innervates about the same number of muscle fibers, *i.e.* the motor units are of roughly uniform size.

Adult soleus motor neurons can, however be induced to increase the number of muscle fibers they innervate if the muscle is partially denervated, *i.e.* if the axons of a portion of the population of the motor neurons are interrupted (Guth 1962, Thompson and Jansen 1977). This expansion in motor unit size constitutes the well-known phenomenon of collateral sprouting which has been shown to occur in many central and peripheral neurons in response to partial denervation (Edds 1953 Raisman and Field 1973). In the case of the adult soleus

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Fig. 1. Summation of twitches from the sprouted nerve and the regenerated nerve in a muscle partially denervated by soleus nerve cut 133 days earlier. Superimposed traces of the twitch to stimulation of the soleus nerve alone (sol.), to the aberrant nerve alone (ab.) and to stimulation of both nerves simultaneously (ab. + sol.). The setting of the stimuli was adjusted so as to give the maximal twitch. The aberrant nerve to this muscle contained 3 motor units.

Muscles partially denervated by nerve cuts

When partial denervation was achieved by cutting the soleus nerve, reinnervation of the soleus muscle was first observed at day 12 after partial denervation. Six rats were examined 16 to 133 days after such partial denervation in order to determine the extent of hyperinnervation and the sizes of motor units.

The degree of hyperinnervation in these muscles was determined by two procedures. First, the summation of twitch tensions to the aberrant and the regenerated soleus nerve was measured. A typical result of this type of measurement is illustrated in Fig. 1. Here it can be seen that the tension generated by the two nerves stimulated together is approximately equal to the sum of the tensions when they were stimulated separately. Such a result was the case for all 6 muscles examined (Table 1), and this result suggests that the aberrant nerve and the soleus nerve innervate different populations of muscle fibers in the muscle.

A more accurate way to estimate the degree of hyperinnervation in these muscles is to sample the innervation of individual muscle fibers by microelectrode recording. This method also has the advantage that it will reveal the existence of any innervation which might be too weak to elicit contraction in the muscle. The end-plate potentials (epp s) from the sprouting and regenerating axons were found only near the center of each muscle fiber at the site of the old denervated end-plates. With a few exceptions, muscle fibers gave epp s only to one of the nerves (*cf.* Table I). Only 2 of 92 fibers in 5 muscles had epp s to stimulation of both the aberrant and the soleus nerves. Hence, both tension measurements and intracellular recordings indicate that the regenerated and the sprouted nerves innervated, with rare excep-

TABLE I Hyperinnervation of muscles partially denervated by nerve cut.

Survival time (days)	Tension measurements						Epp recordings		
	Aberrant N.		Soleus N.		Ab N. + Sol. N.	convergence	No. of fibers with sol. epp.	No. of these also with ab. epp.	convergence
	Tension (g)	Number of units	Tension (g)	Number of units					
16	7.1	ca 10	1.1	10	7.7	6.5	30	1	3.3
22	7.6	5	0.6	3	7.9	3.8	18	1	5.6
32	16.6	11	1.3	3	17.4	2.9	12	0	0
37	8.7	3	1.2	4	9.2	7.6	12	0	0
49	18.2	6	2.1	7	19.1	6.3	—	—	—
133	7.5	3	2.7	10	10.0	2.0	20	0	0

muscle partial denervation causes motor units to increase their size to on the average, abc 3–4 times their normal size (Thompson and Jansen 1977). This increase in size seems t persist indefinitely at least as long as the axons interrupted upon partial denervation do no reinnervate the muscle.

Guth (1962) has reported that when interrupted axons return to partially denervated r soleus or plantaris muscles many of the muscle fibers already innervated by the sprout motor neurons also came to be innervated ("hyperinnervated") by the regenerating axon This observation is interesting since it means that muscle fibers which are innervated ar active accept additional innervation Normally such muscle fibers are refractory to hype innervation (Elsberg 1917)

I have reexamined the problem of reinnervation of partially denervated rat soleus musc and confirm that hyperinnervation does occur although to a limited extent. Furthermore the hyperinnervation is transient Many of the muscle fibers lose their innervation from the sprouted nerve and remain innervated only by the regenerating nerve. Independently Brow and Ironton (1977) and Betz (1977) have obtained similar results from other muscles.

Materials and Methods

Animals used in these experiments were a special strai of albino rats (AO strain, MRC Cellular Immunol ogy Unit, Oxford) whose soleus muscles possess a unusual pattern of innervation (Thompson and Jans 1977). In addition to the normal soleus nerve which is a branch of the tibial nerve, the soleus muscle of these rats frequently receive innervation via a second, smaller nerve, the so-called aberrant nerve hich arises as a branch of the plantar nerve. The earlier experiments have established that this aberrant nerve possesses a variable n mber of the normal complement of soleus motor axons which reach the muscle vi this unusual path instead of through the soleus nerve

Soleus muscles in these animals were partially denervated by either resecting, cutting, or crushing the lateral gastrocnemius–soleus nerve t the point where it branches from the tibial nerve in the thigh The operations were conducted under ether anesthesia. The separate methods of partially denervating the muscles were selected because they provide for different nerve regeneration times (Frank *et al.* 1975) In the *first method the nerv was cut with a pair of dissecting scissors. In the second method* the nerve was crushed several times between the smooth tips of Dumont umber 5 w tchmaker's forceps. The third method consisted of resecting a 1 mm piece of the nerve and ligating the pro mal stump with 9–0 surgical thread. Wounds were sutured closed and a local anesthetic and penicillin (150 000 units/kg, i.m.) administered

For the acute experiments, the muscles nd their nerves were dissected under microscope in oxygenated Ringer's sol tion (composition in mM N Cl, 137 KCl, 5 $CaCl_2$, 10 $MgCl_2$, 1 glucose 10- TrisCl, 10 buffered to pH 7 4). After dissectio the muscle/nerve preparatio was removed to a chamber through which oxygenated Ringer's was perfused (rate ca. 1 ml/min). Isometric tensions were measured by attaching the distal tendon by 6–0 silk suture t sensitive stra gauge (0.15 V/g) The length of the muscle was adjusted t obtain the maximum twitch tension. Nerves were stimulated through suction electrodes. Muscles were directly stimulated by 1 msec duration p lses delivered through silver electrodes on each side of each muscle Evoked synaptic potentials were measured n curarized muscles (2–6 10– g/ml D-tubocurarine) by intracellula micropipettes (20–50 MΩ, potassium acetate filled)

After physiological examination, most of the partially denervated muscles were prepared for histology by fixation in 2% paraformaldehyde–2.5 glutaraldehyde and post f xation 1 osmium tetroxide. The muscles were embedded in plastic and 3 μ thick cross-sections were made through the central region and stained with toluidine blue. The number of muscle fibers n the muscle cross-sections was counted on photographic montages.

Results

The result of the reinnervation of partially denervated soleus muscles differed according t the time of arrival at the muscle of the regenerating motor axons.

TABLE II. Hyperinnervation of muscles partially denervated by nerve crush.

Interval (days)	Tension measurements						Action potentials			End-plate potentials		
	Ab Nerve		Sol Nerve		Ab + Sol tension (g)	% convergence	Sol N	Sol & Ab	% convergence	Sol N.	Sol N + Ab N.	% convergence
	Tension (g)	No. of units	Tension (g)	No. of units								
9	5.9	9	1.9	>14	6.7	16	—	—	—	18	5	28
9	4.3	5	3.4	19	6.6	17	20	3	15	18	3	17
10	2.6	2	5.8	>19	7.1	18	32	6	19	20	3	15
9	4.2	7	2.4	16	5.4	22	11	2	18	28	2	7
7	8.2	6	12.1	20	17.5	16	—	—	—	27	0	0
8	1.4	1	13.2	26	13.9	5	21	1	5	—	—	—
10	4.8		17.9	20	20.0	12	20	1	5	18	0	9
8	12.1	8	10.7	19	18.6	23	—	—	—	33	1	3
8	8.3	6	12.5	>20	16.8	24	47	0	0	33	0	0
11	6.4	4	11.4	>20	15.4	16	26	1	4	10	0	6

the muscle. In addition to the procedure shown in Fig. 2, the muscle was also stimulated directly by currents passed between two electrodes on each side of the muscle in order to determine the maximal twitch the muscle was capable of generating. The ratio of each increment in twitch tension to the maximal direct tension is taken to be a measure of the fraction of the muscle fibers in the muscle innervated by each motor axon. Each fraction was then converted into a number of muscle fibers by multiplying by the number of muscle fibers counted in a cross-section of the muscle.

The results of these unit size measurements for the muscles partially denervated by lateral gastrocnemius-soleus nerve cut are shown in the histogram of Fig. 3. The units from the 6 muscles had an average unit size of 517 fibers. For comparison, the results are also shown from 8 muscles in which there was no soleus nerve reinnervation 19–67 days after partial denervation (reinnervation was prevented in these muscles by resecting a portion of the nerve and ligating the proximal stump). Clearly there is no discernible difference in the unit sizes. Hence, the sprouted aberrant motor units are not appreciably decreased in their expanded size upon reinnervation by the soleus nerve. The regenerating axons seem to have preferentially innervated muscle fibers which had not been claimed by the sprouting axons.

Muscles partially denervated by nerve crushes

In a second group of rats partial denervation of the soleus was achieved by crushing the lateral gastrocnemius-soleus nerve with forceps. This procedure allowed for more rapid reinnervation by the interrupted soleus nerve axons. Strong reinnervation was noted at 9 and 10 days post-partial denervation in 4 of these animals, weak reinnervation was seen in one rat examined at 7 days. Twitch tensions were measured in these muscles and once again the degree of convergence was only moderate. The values are given in Table II, and they were between 5 and 25%.

However, when action potentials and end-plate potentials were recorded in these muscles, a considerable hyperinnervation was detected at early times. In 4 crushed muscles examined

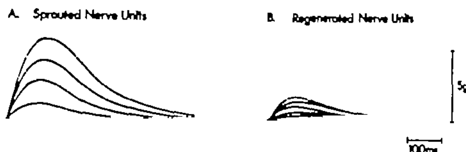


Fig. 2. Twitches of sprouted (aberrant) nerve motor units (part A) and regenerated (soleus) nerve motor units (part B). The muscle was partially denervated by crush of the soleus nerve 71 days earlier. The aberrant and the soleus nerve were in turn stimulated with shocks of carefully graded intensity while measuring the isometric tension generated by the muscle. Each all-or-nothing increment in the twitch tension produced by the activation of a motor axon in the nerve was recorded on the face of a storage oscilloscope. The stimulus intensity was adjusted several times to ensure reproducibility of the responses. Part A shows the 4 units in the aberrant nerve. Part B shows the 5 units of lowest threshold in the soleus nerve.

tions, separate fibers in these muscles. It should also be noted that the two doubly innervated fibers were found in the animals with the shortest survival times.

To determine whether reinnervation changed the sizes of the sprouted motor units, we examined the motor unit sizes of aberrant motor axons in these partially denervated muscles with soleus reinnervation and compared them with the aberrant motor unit sizes in partially denervated muscles without soleus reinnervation. Motor unit sizes were determined using the procedure described previously (Thompson and Jansen 1977). Fig. 2 provides an illustration of this procedure for an adult muscle partially denervated 71 days previously. Fig. 2 A shows the tension increments for the 4 units present in the aberrant nerve. 2 B shows the tension increments for the 5 units of lowest threshold in the soleus nerve which had regenerated to

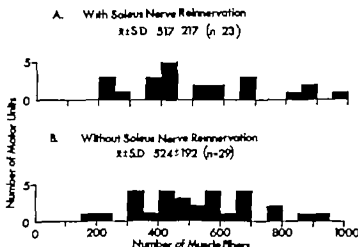


Fig. 3. Sprouted nerve motor unit sizes with (A) and without (B) soleus nerve reinnervation. Part A shows 23 aberrant nerve motor units obtained from 6 muscles 16–133 days after partial denervation by soleus nerve cut. In each of these 6 muscles, there was extensive soleus nerve reinnervation. Part B shows 29 aberrant nerve motor units obtained from 8 muscles 19–67 days after partial denervation. In these muscles partial denervation was accomplished by resection and ligation of the soleus nerve which did not reinnervate the muscle.

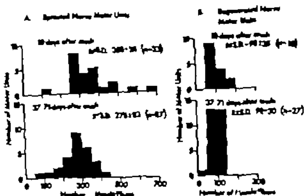


Fig. 5 Sizes of sprouted and regenerated motor units in muscles partially denervated by crush of the soleus nerve. The data are divided into two histograms according to survival time after nerve crush. Top histograms in both A & B give the motor unit sizes for 4 muscles examined 9–10 days after nerve crush; the lower histograms give the motor unit sizes for 6 muscles examined 37–71 days after nerve crush. Motor unit sizes in the data presented here were computed using 3 400 fibers for the normal number present in such muscles. From muscle fiber counts made of some of the muscles, an average of 3 340 was obtained.

the muscles where reinnervation by the soleus nerve was either prevented or delayed about one week longer (averages of 574 and 517 fibers, respectively; see Fig. 3). Apparently if regenerating nerve fibers reach the muscle soon after partial denervation they are able to compete with the sprouting nerve fibers for innervation of the available, denervated muscle fibers. However, the regenerating soleus nerve motor units were never able to regain their normal size. The soleus motor units after regeneration through the crush ($\bar{x} \pm$ S.D. $= 94 \pm 32$, Fig. 5 B) are significantly smaller (Wilcoxon, $P < 0.001$) than the normal soleus motor units (150, Thompson and Jansen 1977).

Further, the measurements of the sizes of the sprouted motor units indicate that these units may be decreasing in size with time after partial denervation. The sprouted motor units are larger at 9–10 days after partial denervation than they are at later times (37–71 days). This difference is statistically significant (Wilcoxon, $p < 0.01$), and suggests that some of the sprouting axons which have been fully functional lose in the competitive interactions with the regenerating axons. Presumably this applies to the muscle fibers which were doubly innervated at early times. This inference is further supported by the finding, shown in Fig. 5 B, that the motor units of the regenerating nerve do not significantly change in size.

During the course of these experiments, a potential complication in this type of experiment was discovered which could severely distort the interpretation of the results. In a number of experiments stimulation of the soleus nerve close to the muscle (distances of 2–5 mm from the muscle) gave a vigorous contraction of the muscle whereas more distant nerve stimulation gave no response. The responses to close stimulation were clearly not due to stimulus spread to the aberrant nerve, as they were abolished once the soleus nerve was removed from the stimulating suction electrode. Further characteristics of this type of soleus nerve response are illustrated in Fig. 6. As shown in part A, the soleus nerve evoked a twitch of amplitude and rise time equal to that of the aberrant nerve twitch. Further, when the soleus nerve and the aberrant nerve were stimulated together, the muscle gave a twitch response which was no



Fig. 4 Intracellular recording from a muscle fiber innervated by both the sprouted (aberrant) nerve and the regenerated (soleus) nerve. The fiber was in a muscle partially denervated by soleus nerve crush 9 days earlier. D-tubocurarine 2 × 10^{-7} g/ml.

9 and 10 days after partial denervation from 7 to 28% of the muscle fibers which gave an epp to the aberrant nerve also gave an epp to stimulation of the regenerated soleus nerve (Table II). An example of one such recording can be seen in Fig. 4. In contrast only one such doubly innervated fiber was found in muscles examined after long survival times. Comparable results were obtained by examining the frequency of action potentials elicited by stimulation of the two nerves in non-curarized muscles (Table II). Thus, it appears that at early times after partial denervation a moderate number of muscle fibers accept innervation from both nerves and that at later times most of this hyperinnervation disappears. There is not a similar trend for hyperinnervation as measured by muscle twitches (Table II). This could have a number of explanations. The intracellular recording measurements give estimates of hyperinnervation only in that portion of each muscle reinnervated by the soleus motor neurons. This portion on the average was only ½ of the total tension in the 9 and 10 day partial muscles (Table II). Assuming a 20% hyperinnervation in this portion of the muscle, only 1/10 or so of the total muscle would be hyperinnervated. This small degree of hyperinnervation may well be beyond our limits of detection in tension occlusion measurements, especially since we normally measure a small degree (up to 14%) of "apparent convergence" in normal adult muscles with aberrant and soleus nerves where in fact no convergence exists (Thompson and Jansen 1977). This inaccuracy in our measurement technique is apparently due to a lack of pure isometric conditions.

In addition to determining whether muscle fibers became innervated by both aberrant and soleus nerve fibers, we also determined by microelectrode recording and grading the stimuli to each nerve independently whether multiple axons in each made synapses onto the individual muscle fibers. Of 216 muscle fibers giving an epp to the aberrant nerve, none were polyneuronally innervated by the aberrant nerve. Hence, as previously reported (Thompson and Jansen 1977), the aberrant nerve fibers form single synaptic contacts upon sprouting. Of 330 muscle fibers giving an epp to the regenerating soleus nerve, only 14 fibers were polyneuronally innervated by the soleus nerve fibers. Even at the earliest times after soleus nerve regeneration only 4 of 93 fibers examined were found to be polyneuronally innervated. Hence, in contrast to the reinnervation of neonatal soleus muscle (Brown *et al.* 1976) and the reinnervation of an adult fast muscle of the rat (McArdle 1975), polyneuronal reinnervation of the soleus muscle is rare.

The sizes of the motor units in the muscles partially denervated by nerve crush were determined as described above and the results are presented in Fig. 5. The material has been divided according to the survival time following partial denervation. The sprouted motor units after nerve crush (Fig. 5 A), average size 315 fibers, did not become as large as those

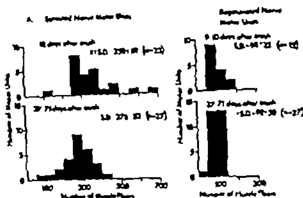


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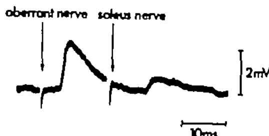


Fig. 4 Intracellular recording from a muscle fi[illegible] innervated by both the sprouted (aberrant) nerve [illegible] the regenerated (soleus) nerve. The fiber was [illegible] *muscle partially denervated by soleus nerve cr[illegible]* 9 days earlier D-tubocurarine 2 [illegible] 10[illegible] g/ml

9 and 10 days after partial denervation, from 7 to 28 [illegible] of the muscle fibers which gave an e[illegible] to the aberrant nerve also gave an epp to stimulation of the regenerated soleus nerve (Ta[illegible] II) An example of one such recording can be seen in Fig 4 In contrast only one such dual[illegible] innervated fiber was found in muscles examined after long survival times. Compara[illegible] results were obtained by examining the frequency of action potentials elicited by stimulat[illegible] of the two nerves in non-curarized muscles (Table II) Thus, it appears that at early tim[illegible] after partial denervation a moderate number of muscle fibers accept innervation from bo[illegible] nerves and that a later times most of this hyperinnervation disappears. There is not a simil[illegible] trend for hyperinnervation as measured by muscle twitches (Table II) This could have [illegible] number of explanations. The intracellular recording measurements give estimates of hype[illegible] innervation only in that portion of each muscle reinnervated by the soleus motor neuro[illegible] This portion on the average was only ½ of the total tension in the 9 and 10 day surviv[illegible] muscles (Table II). Assuming a 20% hyperinnervation in this portion of the muscle, o[illegible] 1/10 or so of the total muscle would be hyperinnervated. This small degree of hyperinner[illegible] vation may well be beyond our limits of detection in tension occlusion measureme[illegible] especially since we normally measure a small degree (up to 14 [illegible]) of "apparent convergen[illegible] in normal adult muscles with aberrant and soleus nerves where in fact no convergence ex[illegible] (Thompson and Jansen 1977). This inaccuracy in our measurement technique is apparen[illegible] due to a lack of pure isometric conditions.

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pparent exception to the rule that innervated and functionally active muscle fibers do not ccept further innervation (Elsberg 1917). However it appears that the sprouting (Thompson ad Jansen 1977) and regenerating (Gutmann and Young 1944) nerve fibers form their ynapses at the old, denervated endplates on the rat soleus muscle fibers. These endplate ites have been shown to retain a special innervatability even on muscle fibers which are ully active (Jansen et al. 1973) or even innervated elsewhere (Frank et al. 1975). The re generating nerve fibers of the present material appeared to form their synapses adjacent to a sprouted nerve terminal within the old endplate site; thus, the hyperinnervation is in line with previous observations. Bennett and Raftos (1977) have reported that sprouting nerves in axolotl muscles form synapses at denervated endplates which do not fully occupy the original site. These sites can then be reinnervated also by regenerating nerve fibers. This could be the case in the present experiments also.

The present results indicate that the amount of hyperinnervation is related to the time elapsed between partial denervation and reinnervation by the regenerating axons. If the regenerating axons began forming synapses in the muscle before the sprouting nerve fibers had consolidated their new end-plates, as after nerve crush, then a substantial number of the muscle fibers became hyperinnervated. If however regeneration occurred later after the sprouting nerve fibers had completed expansion, as after nerve cut, then very few muscle fibers became hyperinnervated. A simple explanation of this time dependence would be that the terminals of the sprouting nerve fibers require a certain period of occupancy of the endplate site before they can render the site refractory to further innervation, perhaps by growing completely over the synaptic area (Bennett and Raftos 1977) or perhaps by rendering any empty synaptic space non-innervatable (Frank et al. 1975). There is, however an inconsistency to this otherwise appealing view. Guth (1962) reported a much higher incidence of hyperinnervation in his partially denervated soleus muscles than what was found in the present material. In Guth's experiments the partial denervation was produced by spinal root section. His reinnervation would therefore be appreciably later than what we found after distal nerve section. Guth, however assessed the degree of hyperinnervation solely from tension measurements. As noted above there are potential complications with these measurements. Otherwise, I cannot explain the discrepancy between Guth and the present results.

The hyperinnervation was significantly reduced over a time course of less than one month. This means that one of the synaptic inputs on the hyperinnervated fibers was eliminated and suggests that the two nerve terminals interacted competitively for maintenance on each muscle fiber. The finding that the sprouted (aberrant) motor units significantly decreased in size during the time period over which the elimination occurred, while the regenerated motor units did not change in size, further suggests that it is the terminals of the sprouted nerve fibers which were losing out in the competition. It may well be that the expanded, large size of the sprouted motor units places their nerve terminals at a competitive disadvantage in relation to the regenerating axons, which support many fewer terminals in the muscle. A similar competitive advantage of small motor units over large motor units has been noted in the elimination of polyneuronal innervation from soleus muscle fibers of neonatal rats (Brown et al. 1976). Brown and Ironton (1977) have described cases of partial denervation of the mouse peroneus tertius muscle where a few remaining axons sprout to innervate all the

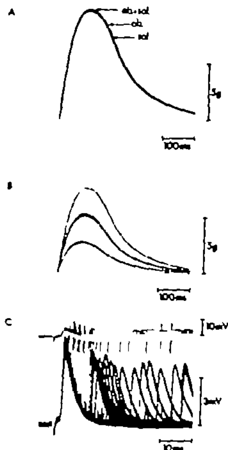


Fig. 6. Illustration of sprouting of aberrant nerve fibers in the soleus nerve distal stump. A segment of the soleus nerve had been resected 59 days earlier. Part A shows the twitches obtained to stimulation of the soleus nerve alone (sol.), the aberrant nerve alone (ab.), and both nerves together (ab. + sol.). The twitches to each nerve are almost indistinguishable and occlude completely. Part B also shows the 3 units which were found in the soleus nerve close to the muscle; the aberrant nerve contained 3 identical units. Part C shows a recording of an endplate potential from a single fiber of the curare (4 × 10⁻ g/ml) muscle. Stimulation of the aberrant and soleus nerves gave end-plate potentials which were indistinguishable in rise time and amplitude. In part C the aberrant nerve is stimulated first and the soleus nerve at a variable latency later. If the soleus nerve stimulation occurred within 7 msec of the aberrant nerve stimulation, no response was obtained to the soleus nerve stimulus. Stimulation of the peripheral end of the original soleus nerve was effective directly to the muscle while its central portion was inexcitable.

larger than when either nerve was stimulated alone. The number and sizes of the units in the soleus nerve were the same as those in the aberrant nerve (Fig. 6 B). Finally, microelectrode recordings from the muscle fibers revealed that each fiber in which an action potential (or epp) was evoked by stimulating one nerve also had an action potential (or epp) evoked by the other nerve. Further, as shown in Fig. 6 C, the epp's occluded each other if the second stimulus followed the first by less than about 7 ms. This is in contradistinction to the hyperinnervated muscle fibers shown above, where epp's to the two nerves summated and could not be occluded. Therefore, in these experiments the cut nerve had apparently not regenerated to the muscle. Rather, sprouts of the aberrant nerve fibers had entered the peripheral stump of the soleus nerve and were growing back in a proximal direction. It is well-known that the sheaths, tubes, and perhaps products of degeneration of isolated nerve segments form an excellent attractant and conduit for nerve growth (Ramon y Cajal 1959). This appears to be the explanation for the present finding.

Discussion

The results confirm the observations of Guth (1962) that nerve fibers regenerating to a partially denervated rat soleus muscle can hyperinnervate muscle fibers which are already functionally innervated by sprouted nerve fibers. These results, therefore, constitute an

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denervated fibers. Nevertheless, upon regeneration of the interrupted axons, some of the muscle fibers initially become dually innervated. Subsequently, the dual innervation is lost by elimination of the sprouted nerve terminals and there is a reappearance of muscle fibers innervated exclusively by the regenerating axons. What may be similar cases of competitive disadvantages of sprouted nerve terminals in relation to regenerating nerve terminals have been reported by Betz (1977) in a rat foot muscle, by Roper (1976) in a parasympathetic ganglion of the frog, by Cass *et al* (1973) and Bennett and Raftos (1977) in axolotl muscle, and by Guth and Bernstein (1961) in a mammalian sympathetic ganglion.

The elimination of hyperinnervation which occurs in these muscles may be contrasted with the lack of a similar elimination observed by Frank *et al* (1975). Frank *et al* transplanted foreign nerves to the proximal end of the soleus muscle. These foreign implants established synapses at ectopic sites on the muscle fibers when the original innervation of the muscle was interrupted. If the interrupted axons regenerated quickly, they were able to reinnervate almost all of the muscle fibers by establishing synapses at the old endplates. As a result, many muscle fibers were hyperinnervated by the foreign nerve at an ectopic site and by the original nerve at the old endplate. This hyperinnervation persisted indefinitely. Two possible reasons can be offered for the elimination of hyperinnervation seen in the present study. First, it could be that the interactions necessary for synapse elimination can occur only for a limited distance along each muscle fiber, as suggested by the experiments of Kuffler *et al.* (1977). In the present experiments the new synapses were all formed at or very close to an original endplate. In the experiments of Frank *et al* on the other hand, the synapses were located several mm apart, perhaps too far for the competitive interaction to act. A second, alternative explanation of the difference would be that the competition in the present experiments was for occupancy of a defined synaptic area (the old endplate site), while in the experiments of Frank *et al* there was no such competition.

I wish to thank Jan Jansen for his contributions of ideas and guidance during this study. Additional thanks go to Damien Kuffler for valuable discussions, Håvard Tønnesen for technical assistance and Ingrid Knutsen for preparing the histological sections. I was supported by a NATO postdoctoral fellowship from the U.S. National Science Foundation.

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e third ventricle (*cf* Andersson 1977). Therefore, it has been of interest to cover this area a field of bipolar electrical stimulation in the conscious goat. The use of thermo-couple ectrodes has made it possible to study also water intake during moderate, controlled ating within the medial forebrain. This has been of interest primarily because more tense heating of this part of the brain earlier was found to cause drinking in the goat Andersson and Larsson 1961).

Methods

nimals. Local forebrain warming was made in 13 female goats (b t 35 kg). Four of these animals were so used for electrical stimulation. The goats were kept in metabolism cages at room temperature (20 ± 1°C), here all the electrical stimulations and most of the heating experiments were conducted. The animals ceived hay *ad lib* and had their water buckets refilled at 8 am every day. The experiments were started ound 10 am when the goats had consumed hay and fresh water to satisfaction.

Electrode implantation. All goats had pair of stainless steel thermo-couple electrodes (d 0.7 mm) ermanently implanted through the septum into the preoptic anterior hypothalamic region for the final urpose of making radio-frequency (RF) lesions. The implantations were performed under general (nembutal) anesthesia about 2 weeks before any experiment were started. The twin electrodes were either placed long the midline of the brain with an interspace of 3 to 5 mm, or bilaterally with an interspace of 3 or 4 mm. The length of the uninsulated tips of the electrodes varied between animals from 3 to 5 mm.

Electrical stimulation. A modified Hess' technique, earlier described in detail (Andersson, Persson and 1969) was employed. Bipolar as well as unipolar brain stimulations were performed. In the latter subcutaneous steel-wire on the animal's neck served as the reference electrode. The following timulus parameters were used. Current strength 0.4–4 mA, Pulse frequency 50 s, and Pulse duration 1.5 ms. The stimulation periods were 1 to 10 min.

Radio-frequency (RF) warming. A steady 2 C elevation of the temperature of the brain tissue between the electrodes was induced by applying RF-energy between the uninsulated ends of the thermo-couple electrodes, having their temperature recording units connected to an "Ellab" thermometer. The duration of he warming periods was 40 min. For further details about the RF-technique employed see Gale (1963).

Hydration. Occasionally electrical and thermal stimulations were performed in the pre-hydrated goat about 90 min after the administration of 60 or 100 ml/kg of 38°C water into the rumen by stomach tube).

Urine sampling. During experiments in hydrated animals urine was collected via a retention catheter for determinations of flow and osmolality (by use of an "Advanced Instruments Inc" osmometer).

Histology. At the termination of the experimental series involving electrical and thermal stimulation, the electrodes were used for producing RF-lesions in the medial forebrain of all goats. At the end of the post-lesioning observation periods the animals were decapitated under nembutal anesthesia and the heads were perfused with physiological saline followed by 8% formol saline. After formol fixation block of the brain (including the preoptic region, the thalamus and the hypothalamus) was embedded in celloidine and cut by serial transverse sectioning at 30 μm. The sections were stained either with toluidine blue or according to Loyez (*cf* Culling 1957).

Results

Electrical stimulation

Of the 4 animals subjected to electrical stimulation, 2 goats (here named A and B) had bilaterally implanted electrodes (3 mm apart, uninsulated ends 3 mm). On preliminary bipolar stimulation both animals characteristically responded with post-stimulatory water consumption, and their dipsogenic responses were subjected to continued analysis over periods of 5 and 3 weeks respectively. The other two animals had their electrodes placed along the midline of the brain. Here bipolar electrical stimulation did not induce drinking. However in one of these goats (goat C) strong (4 mA) unipolar stimulation via the anterior electrode (Fig. 3) elicited drinking after a latency time of bout 30 s. Since

Acta physiol. scand. 1978. 103. 92–99
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Delayed drinking in response to electrical and thermal stimulation of the medial forebrain

By

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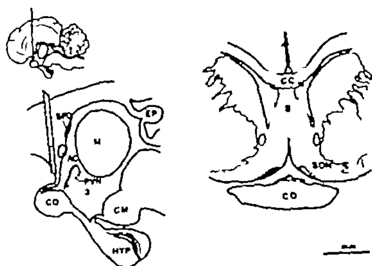
Received 28 November 1977

Abstract

RUNDGREN M *Delayed drinking in response to electrical and thermal stimulation of the med. forebrain* Acta physiol scand 1978 *103* 92–99

Water intake in response to electrical and thermal stimulation of the medial forebrain was studied in the goat. When the frontal wall of the third cerebral ventricle was included in a field of bipolar electrical stimulation a dipsogenic response was obtained after discontinuation of the stimulation. Release of antidiuretic hormone (ADH) was apparently also elicited. The water intake was roughly proportional to the amount of current which had been applied during the stimulation period Water consumption in response to stimulation attenuated the dipsogenic effect of subsequent stimulation as did also pre-stimulation hydration by stomach tube. A 2°C elevation of the temperature of parts of the preoptic/anterior hypothalamic region for 40 min periods induced cumulative drinking starting after 5 to 18 min. There were great interindividual differences in the amount of water consumed in response to the thermal stimulation, possibly due to variations in thermo-couple electrode placement. The dipsogenic effect of forebrain warming was inhibited by pre-hydration, and by lowering of the environmental temperature. The delayed thirst responses are discussed in relation to stimulus-bound drinking previously observed in the same and other species. It appears possible that the delayed drinking was a manifestation of artificially induced excitation of juxtaventricular "thirst" receptors.

Verney's (1947) fundamental studies in conscious dogs conclusively demonstrated that a cerebral sensory mechanism plays an important role in the control of water balance by regulating the release of antidiuretic hormone (ADH) from the neurohypophysis. His investigations also implied that the sensitive brain cells primarily are excited by a reduction of their own volume and are located in the anterior hypothalamus. This osmoreceptor concept was extended to involve also the regulation of water intake when it was found that injections of hypertonic NaCl into the anterior hypothalamus elicited excessive drinking in water-replete goats (Andersson 1953). Electrical stimulation was later employed to study the cerebral thirst mechanism, and it was found that strictly stimulus-bound drinking could be elicited by unipolar or bipolar stimulation within the perifornical region in the goat (Andersson and McCann 1955), and the lateral hypothalamus of the rat (Greer 1955). However the integrated results of later studies imply that hypothalamic receptors regulating water intake and ADH-release are located more medially close to the anterior border of



g. 2. Position of the bilaterally implanted electrodes in goat A shown on midsagittal (*left*) and transverse (*right*) sections. The 3 mm uninsulated ends of the electrodes are hatched. AC anterior commissure, CC corpus callosum, CM corpus mamillare, CO chiasma opticum, EP epiphysis, HYP = hypophysis, MI massa intermedia, PVN = paraventricular nucleus, S septal region, SFO subfornical organ, SON supraoptic nucleus, 3 third ventricle.

strength was then generally limited to 0.5 mA. When goat B was not pre-hydrated (6 sessions) the initial trials (0.5 mA for 5 or 10 min) invariably induced drinking (200 to 400 ml) about one min after the stimulation had been switched off. Repeated stimulations of the same strength and duration did not induce further water consumption. Likewise, prehydration (2 sessions) prevented post-stimulatory drinking.

Apparent release of antidiuretic hormone (ADH). The bipolar stimulations which caused delayed drinking apparently also resulted in release of ADH as indicated by the results shown in Fig. 1 (right). Here goat A had developed positive renal free water clearance" in consequence of over-drinking after preceding periods of stimulation (Fig. 1 left). Renewed stimulations then caused temporary inhibition of the water diuresis and the degree of inhibition was proportional to stimulus duration. Similar graded, antidiuretic effects were obtained when the animal had been hydrated by stomach tube before the experimental session started.

The threshold stimulus (0.5 mA) for delayed drinking in goat B was ineffective, not only in eliciting drinking, but also in inhibiting the water diuresis when this animal was pre-hydrated. However an antidiuretic effect could then be obtained in response to more intense stimulation (1 mA for 5 min).

Electrode location. The position of the electrodes in goat A is shown in Fig. 2. It is evident that electrical impulses passed between the uninsulated ends of the electrodes must have affected brain tissue close to the anterior border of the third ventricle. This is also indicated by the fact that the RF-lesion later induced in this goat included about two thirds of the anterior wall of the ventricle and extended 2 mm in front of the anterior



Fig. 1 *Left* Cumulative post-stimulatory drinking in response to repeated electrical stimulations in goat A Note the rise in stimulus threshold for drinking after previous water consumption. *Right:* Stimulation causing temporary inhibition of the water diuresis which had developed in consequence of the previous cumulative drinking. No drinking occurred in response to the relatively strong stimulation when the animal was hydrated to this extent No stimulations were performed during the interval (10 30-11 30) Current strength and duration above each stimulation period (S). L = Licking in the water

stimulation at this strength also induced restlessness further studies of the effects of electrical stimulation were omitted

Drinking after bipolar stimulation Goat A was subjected to consecutive electrical stimulations on 17 daily sessions over a period of 5 weeks. When not pre-hydrated (15 days) the lowest intensity of stimulation needed to induce the initial thirst response was either 1 mA for 5 min, or 2 mA for 1 min. Preceding stimulations of lower strength and/or shorter duration (*e.g* 0.5 mA for 10 min, or 1 mA for 1 min) were found ineffective. Drinking generally commenced precisely half a min after the discontinuation of stimulation and $1\,430 \pm 210$ ml of water was then drunk in one sequence. On repeated trials in the same session stronger stimuli were needed to induce further drinking as demonstrated in Fig. 1 Here, and in other sessions, the goat did no longer drink after intense (3 mA, 1 and 5 min) stimulation when preceding stimulations had induced a total water intake of about 4 l Similarly during the two sessions when goat A was pre-hydrated (water load 60 ml/kg) the stimulus threshold for drinking was elevated The duration of post-stimulatory thirst was studied on two occasions by temporarily preventing the animal from drinking. The experiments revealed that the urge to drink was still present 9 min after stimulation

In *goat B* bipolar stimulation at 1 mA, or stronger induced restlessness and shivering For that reason the animal was only subjected to 8 experimental sessions and the stimulus

s performed when the goat was either pre-hydrated (n = 2), or transferred to a cold 4 C) environment 1 h prior to the start of central warming (n = 2). Under these circum-nces no water was consumed. Furthermore, when the animal was pre-hydrated 0 ml/kg) the forebrain warming obviously did not cause significant release of ADH, ce no reduction of the water diuresis occurred.

Electrode location. In goat C the electrodes were placed along the midline of the brain. e position of their 5 mm uninsulated ends is shown in Fig. 3. It appears that most of e anterior wall of the third ventricle had been subjected to warming during the application RF-energy sufficient to cause a 2 C elevation of the temperature at the tips of the ectrodes. An idea about the approximate extent of the warming was provided by later F-lesioning (raise of temperature to 60 C for 4 min) which resulted in the destruction of most the entire anterior wall of the ventricle including most of the subfornical organ and e supraoptic crest. The bilateral extent of this lesion, which made the animal adipsic, is own to the right in Fig. 3.

Discussion

rinking as effect of electrical stimulation at various sites in the hypothalamic region has een reported in several mammalian species over the past 22 years (*cf.* Fitzsimons 1977). enerally the effective sites of stimulation have been at the sagittal level of the descending olumn of the fornix, or further laterally and the dipsogenic effect has been rather strictly timulus-bound. In the goat (Andersson and McCann 1955) drinking as effect of peri-ornical stimulation was not attenuated by water consumption. Therefore, conspicuous over-hydration could be induced by frequently repeated, or prolonged periods of stimula-ion, and it was assumed that this polydipsia was the manifestation of hyperactivity in a hypothalamic "thirst center". However the results of recent studies imply that cerebral eceptors regulating water intake predominantly are located more medially in, or close o the anterior wall of the third ventricle (*cf.* Andersson 1977). For this reason it appears nlikely today that the stimulus-bound drinking previously obtained at more lateral sites in he hypothalamic region was due to excitation of cerebral "thirst receptors". A more probable explanation seems to be stimulation of pathways transmitting impulses from receptor areas to parts of the brain where the urge to drink is integrated. Some support for this view is provided by the results of the present study.

Stimulus-bound drinking was not elicited when the presumptive cerebral "thirst receptor" area was subjected to field-stimulation. Rather unexpectedly however thirst became apparent half to one min after cessation of stimulation. When not satisfied, the urge to drink persisted for several minutes. Furthermore, like normal thirst, it was inhibited by dilution of the body fluids (pre-hydration). Apparently temporary field-stimulation of brain tissue bordering the third ventricle in some manner induced post-stimulatory activity similar to that developing in this area during dehydration. However it is impossible to tell whether this activity was a manifestation of events at the receptor level, or consisted of post-stimulatory epileptic discharge in neurons normally processing receptor information. Of interest with regard to the latter possibility are reported effects of electrical stimulation within the dorsal vagal nucleus in the goat (Andersson, Kitchell and Persson 1958). Normal

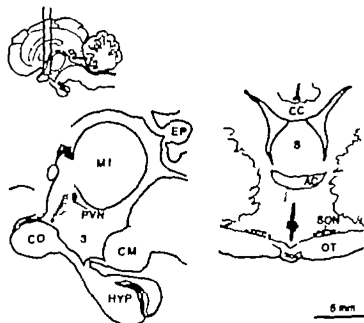


Fig. 3 *Left* Midsagittal section showi g the location of the electrodes in goat C (implanted along the midline of the brain). This animal consistently responded with drinking to RF-warming (2 °C) between the 5 mm uninsulated ends of the electrodes (hatched). *Right* Transverse section at the level of the anterior commissure showing the bilateral extent (hatched rea) of the RF-lesion later produced in this animal. *Abbreviations.* See Fig. 2. Additional. OT = optic tract.

commissure. The position of the electrodes in goat B has not yet been verified by histological examination. However judging from X ray pictures taken during the implantation procedure, the uninsulated ends of the electrodes had a location very similar to what is shown in Fig. 2. An indication that the stimulations in goat B involved a cerebral thirst receptor area was provided by the fact that the RF lesioning induced permanent adipsia.

Forebrain warming

Water intake Prior to RF-lesioning all animals were subjected to a preliminary expt in which the effects of local forebrain warming (C for 40 min) were studied. As expected from the studies by Magoun *et al* (1938) and later investigations of cerebral control of body temperature, all animals reacted with peripheral vasodilatation and polypnea of varying degree. 6 of the goats also drank during the first warming period. Since drinking occurred also in additional expts. it appears safe to conclude that it was a true effect of the forebrain warming. However the latency time for thirst varied between animals from 2 to 18 min and there were also great interindividual variations in the amounts of water consumed. Furthermore the water intake was apparently not proportional to the intensity of polypnea.

One of the responding animals (goat C) was selected for a further analysis of the dipsogenic effect When maintained at room temperature, and not pre-hydrated (in expts.) goat C invariably responded with drinking during the 40 min forebrain warming. The average latency time for drinking was 6.7 ± 1.6 min and the mean cumulative water intake was $1\,280 \pm 650$ ml In between these positive expts. identical forebrain warming

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reticulo-ruminal motility was completely arrested during the stimulation period, conspicuous, and rather persistent hyperactivity developed almost immediately after.

Available information on the nature of juxtaventricular receptors involved in the control of water balance (*cf* Andersson 1977) appears of interest considering eventual influences at the receptor level of the present electrical and thermal (see below) stimulations. Cerebral receptors regulating water intake and ADH-release are obviously sodium-sensitive and some recent studies vaguely indicate that Na-transporting enzyme activity forms a link in the excitation process (Olsson, Larsson and Liljekvist 1976, Rundgren *et al* 1977). The possibility exists that the present field-stimulation induced transmembranal redistribution of Na and other cations. Local redistribution of Na could have acted like a physiologic stimulus for thirst and ADH release when the pulse train no longer interfered with the normal function of the brain area in question. This would explain why post-stimulatory thirst was susceptible to the normal inhibitory influence of hydration.

Heating of the preoptic/anterior hypothalamic region via large medially implanted thermodes was earlier found to induce drinking in the goat concomitant with mobilization of heat dissipation mechanisms (Andersson and Larsson 1961). The validity of the observation as evidence for a suggested physiological interaction between cerebral mechanisms regulating body temperature and water intake is obscured by the fact that the thermode temperature was elevated by 5°C or more. Heating to that extent might have acted as a noxious stimulus of structures of importance for the development of thirst. In the present study drinking was elicited by a moderate (2°C) elevation of the medial forebrain temperature, which hardly could have been a noxious stimulus. This, however, provides no additional evidence for an interaction of the kind mentioned above, since there was no correlation between the amounts of water consumed and the degree of polypnea. Other events (*e.g.* blood pressure elevation) may well explain the fact that the dipsogenic effect vanished during acute exposure to external cold.

Why moderate elevation of the temperature in the medial forebrain elicited drinking remains an open question. At present, any attempt to answer it must be highly speculative. However the presumptive cerebral "thirst receptor" area was subjected to warming in the positive expts. (Fig. 3). Due to the van t Hoff effect, the elevated temperature ought to have induced increased enzymatic activity in that area. Provided Na-transporting enzyme activity really is essential for excitation of cerebral "thirst receptors" (see above), the metabolic effect of warming could explain the animals urge to drink.

This work was supported by grants from Karolinska Institutet, and from Svenska Sällskapet för Medicinsk Forskning.

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...LE I. Description of rabbit diets.

Sodium chloride content (%)	Source
0.25	Altromin GmbH Lage, Lippe, West Germany
2.5	Altromin GmbH Lage, Lippe, West Germany
0.37	Astra-Ewos AB, Södertälje, Sweden
2.0	Astra-Ewos AB, Södertälje, Sweden
0.43	Astra-Ewos AB, Södertälje, Sweden

...inary PGs originate within the kidney (Frölich *et al.* 1975, Williams *et al.* 1977) and their ...asurement therefore constitute an index of intrarenal biosynthetic activity. Our results ...ow that sodium deprivation markedly enhances renal excretion of PGs pointing to the ...ssibility of an antinatriuretic role of the PGs.

Material and Methods

...rabbits were nonpregnant albinos weighing 2.5 kg at the start of the study. They were maintained on ...ne of the diets shown in Table I.

The animals were kept in metabolic cages with one rabbit in each cage. The 24-hour urines from each ...bit were collected separately. Special care was taken to avoid degradation of the PGs by collecting ...urine in traps surrounded by dry ice. The urine was then frozen very soon after being voided and kept ...zen until analyzed.

Experimental protocol. Two experimental designs were used. In the first the rabbits were fed the normal ...et (3), high sodium diet (2) and low sodium diet (1) *ad libitum*. On each diet animals were allowed to ...ilibrate for one week and data were then collected for 4 days. It was observed that intake of both food ...d water varied considerably between the high sodium diet and the other dietary regimens. With the low ...dium diets the food intake was 3–4 times that of the high sodium diet. In a second series of expts. ...erefore decided to give the animals different amounts of sodium while keeping the intake of food constant. ...he diets were given in the following order: high sodium diet (4) for 6 days, normal diet (3) for 6 days and ...sodium diet (5) for 6 days. The first 3 days were allowed for equilibration and urine was collected ...nly for another 3 days.

Analysis. Urinary sodium and potassium was measured using flame photometry (Instrumentation ...aboratory Inc. Model 143).

$PGF_{2\alpha}$ in urine was measured by radioimmunoassay (RIA). This procedure has been described in detail ...where (Oliw *et al.* 1978 b). In brief, the precision of the method was evaluated by replicate (n = 6) ...determination of the same samples. The coefficient of variation was determined on 4 separate occasions ...d found to be 10.7 %, 13.4 %, 12.7 % and 18.5 % (mean 13.8 %). The validity of the method was assessed ...by co-determination of 6 different samples with gas chromatography—mass spectrometry (GC–MS) using ...deuterated $PGF_{2\alpha}$ as an internal standard. The results are shown in Table II. It is seen that the values ...btained by the two methods are in close agreement, thus demonstrating the validity of the RIA method.

In experimental design 1 PGE_2 was reduced by $NaBH_4$ to $PGF_{2\alpha}$ and $PGF_{2\beta}$ and $PGF_{2\beta}$ measured by ...RIA using $PGF_{2\beta}$-antiserum (Oliw *et al.* 1978 b). In experimental design 2 PGE_2 was determined ...by bioassay on the rat fundus strip essentially according to Larsson and Änggård (1974). To 20 ml of ...rabbit urine was added 50 000 cpm of ^3H-labelled PGE_2 (100–200 Ci/mmole, New England Nuclear, Darm...stadt, West Germany). After adjusting the pH to 3 using 30–40 drops of 50 % formic acid, the sample ...was extracted twice with 20 ml of $CHCl_3$. The solvent was evaporated to dryness and applied on 1 g

Acta physiol. scand. 1978. 103. 100–106

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The influence of dietary sodium on urinary prostaglandin excretion

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Received 30 November 1977

Abstract

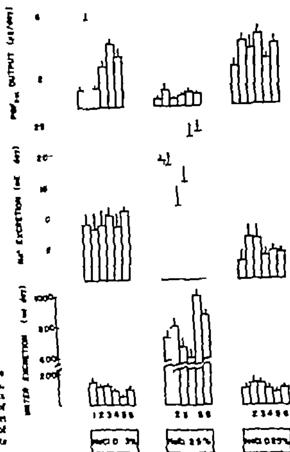
DAVILA D., T DAVILA, E. OLIW and E. ÄNGGÅRD *The influence of dietary sodium on urinary prostaglandin excretion* Acta physiol scand. 1978. *103* 100–106.

The influence of dietary sodium chloride on the urinary excretion of prostaglandins (PGs) was studied in unanesthetized female rabbits housed in metabolic cages. Urinary PG levels were determined by radioimmunoassay bioassay and gas chromatography-mass spectrometry In the first experiment rabbits were fed at high (2.5 %) and later a low (0.25 %) sodium chloride diet *ad libitum*. A 2 5 fold increase in excretion of immunoreactive $PGF_{2\alpha}$ ($iPGF_{2\alpha}$) and $iPGE_2$ was noticed when animals were given the low salt diet. Since it could not be excluded that dietary factors other than sodium chloride contributed to the change a second more controlled experiment was undertaken. Rabbits were fed 30 g/kg per day of diets differing only in the content of sodium chloride, 2 % and 0.37 respectively On the high salt diet the rabbits excreted 0.1 ± 0.04 µg/day of PGE_2 and ...0 ± 0.5 µg/day of $iPGF_{2\alpha}$ After equilibration on the low salt diet the PGE excretion rate increased to 1.5 ± 0.3 µg/day ($p < 0.001$) and that of $iPGF_{2\alpha}$ to 3.4 ± 0.4 µg/day (p ... These results thus point to an inverse relationship between renal sodium excretion and the activity of the renal prostaglandin system.

The role of the prostaglandins (PGs) in the renal handling of sodium is unsettled. Early studies using infusion of E and A types of PG demonstrated natriuretic and diuretic effects (Herzog *et al* 1967 Vander 1968 Lee 1973) Similarly an infusion of arachidonic acid, a PG precursor into the renal artery caused natriuresis in doses which did not alter renal blood flow or glomerular filtration rate (Chang *et al* 1975 Tannenbaum *et al.* 1975 Bolger *et al* 1976) On the other hand meclofenamate increases renal sodium excretion both in the conscious and in the anesthetized dog in response to volume expansion (Kirschenbaum and Stein 1976, 1977 *cf* Oliw *et al* 1978 a) Furthermore, Tobian *et al* (1974) and Tobian and O Donnell (1976) have shown that sodium loading in rats depresses intrarenal concentration of PGE, whereas deprivation has the opposite effect Quite recently Weber *et al* (1977) reported that a high sodium diet depresses the renal excretion of PGE_2 in the rabbit.

In the present study we have attempted to evaluate the effect of the dietary sodium on the renal excretion of PGs in the conscious rabbit. As has been shown earlier the primary

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ig. 1 The effect of dietary sodium on the daily excretion of immunoreactive PGF₂α, sodium and water in 6 different rabbits fed *ad libitum*. The registrations (means of days ± S.E.) of variables on each diet were made after an equilibration period of one week. Figures under the bars denote each individual rabbit

ould possibly alter the renal output of PGs by mechanisms unrelated to renal handling of odium. In the second series of expts. the rabbits were restricted to a food intake of 30 g/kg/day. Water was offered *ad libitum*. The animals were first given the high sodium diet (4) nd then low sodium diet (3) and measurements were made after three days of equilibration n each diet. The results are shown in Fig. 2. Again an inverse relationship was found between PG excretion and sodium output. The values for urinary volume, pH and output of potassium were constant between the two dietary regimens. The changes in IPGF$_{2\alpha}$ and PGE$_2$ excretion were significant (p 0.01 and p 0.001 respectively).

Discussion

The most apparent finding of the present study was that the urinary output of PGs was dependent on dietary sodium. An involvement of renal PGs with the renal handling of sodium was previously suggested by Tobian *et al.* (1974) and by Tobian and O'Donnel (1976). These authors noted an elevation of the intrarenal levels of PGE$_2$ but not of PGF$_{2\alpha}$

TABLE II Comparison of $PGF_{2\alpha}$ determination by radioimmunoassay and gas chromatography-spectrometry in urine.

Sample	RIA ng/ml	GC MS ng/ml	Difference
1	36	36	0
	21	34	38
3	37	37	0
4	20	23	13
5	27	28	4
6	3	39	18
Mean ± S D	29±7	33±6	12±14

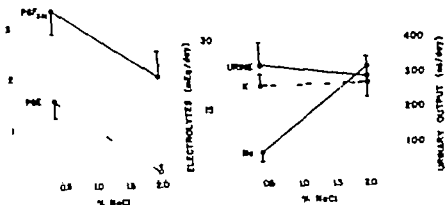
silicic acid (Unisil, 100-200 mesh Clarkson Chem. Comp Inc., Williamsport, Pa., USA) column in 3 ethyl acetate in toluene. After 30 ml has been allowed to pass through the column the PGE_2-like material was eluted using 60 ml of 60 % ethyl acetate in toluene. This fraction was evaporated to dryness, dissolved in 1.5 ml of 96 % ethanol and kept at −20°C until assayed The recovery of ³H PGE_2 through the whole procedure was about 30 % (range 25-57 %) The recovery of added 50 ng of PGE_2 was assessed in 1 and after correction of losses of ³H PGE_2 found to be 98 %. Prior to bioassay the samples were reduced to dryness and reconstituted in 1 ml of Tyrode solution. The assay of the rat fundus strip was carried according to Weeks *et al.* (1968). The validity of the assay was further checked by treatment of the samples with dilute NaOH This procedure, which coverts all PGE-compounds to the respective PGB-compound, resulted in complete biological inactivation The level of the PGE_2 present in the original sample obtained using the data from the bioassay and the recovery of radioactive PGE_2 in each sample. Statistical significance was determined by Student's t-test.

Results

In the first experiment rabbits were fed a normal diet, a high (2.5 %) and a low (0.2% sodium chloride containing diet *ad libitum* The excretion of prostaglandins, sodium and water is shown in Fig. 1 An inverse relationship was found to exist between the excretion of sodium on the one hand and of $iPGF_{2\alpha}$ on the other hand.

When the rabbits were fed the normal rabbit chow diet (5) containing 0.43 % NaCl a fold interindividual variation in output of $iPGF_{2\alpha}$ was seen However the intraindividual variation was relatively small (*cf* Fig. 1) The mean excretion of $iPGF_{2\alpha}$ was 2.9 ± 1.7 µg/24 h (mean ± S D) Change to the higher sodium diet led to a decrease in the renal output of $iPGF_{2\alpha}$ to a mean value of 0 69 ± 0.3 µg/24 h but increased both sodium and water excretion When during the last stage of the expts. the rabbits were eating the diet, with the lowest content of sodium the urinary $PGF_{2\alpha}$ excretion again increased to a mean level of 3.5 ± 0 7 µg/24 h. The mean level of $iPGF_{2\alpha}$ and $iPGE_2$ for the two later diets are shown in Table III The differences on PG excretion between the two diets were statistically significant (both $p < 0\ 001$)

It thus appeared that there might be an inverse relationship between the renal excretion of sodium and the excretion of $iPGF_{2\alpha}$ and $iPGE_2$. However two factors led us to undertake a second more controlled experiment. Firstly the rabbits ate 3–4 times more of the low sodium diet as compared to the high sodium diet. Secondly the rabbits drank roughly 3 times more during the high salt diet as compared to the low salt diet These differences



2. Effect of dietary sodium (2 and 10 mmol/kg/day) on the excretion of PGE_2, measured by two-way [illegible] on immunoreactive $PGF_{2\alpha}$ (top), sodium, potassium and [illegible]ater (bottom). Food intake [illegible] restricted 30 g/kg/day but [illegible]ater [illegible]as offered *ad libitum*. T[illegible] and three asterisk[illegible] denote [illegible] difference between [illegible] two diets at p [illegible] 0.01 and p [illegible] 0.001 respectively. Mean [illegible] S.D.

74), with anesthetized dogs (McGiff *et al.* 1970, Williams *et al.* 1977) and conscious humans [illegible]rolich *et al.* 1975) have demonstrated increased release of PGs following infusion of angio[illegible]sin II. Moreover, direct application of angiotensin II to slices of rat renal papilla *in vitro* [illegible]duces release of PGs into the incubation medium (Danon *et al.* 1975 a). A second pos[illegible]bility could be that the renal medullary arachidonic acid metabolism is sensitive to varia[illegible]ons in ADH or in medullary osmolality as indicated by the work of Danon *et al.* (1975 b), [illegible]ho reported elevated PG output from slices of rat renal papilla in response to hypertonic [illegible]edia. The third and perhaps most interesting alternative would be that the increased [illegible]vels of urinary PGs are [illegible] consequence of their involvement in a tubular sodium conserving [illegible]echanism as has been suggested by Tobian and O'Donnel (1976). Both the increased intra[illegible]nal levels of PGE_2 during low salt intake observed by these authors, and the increased [illegible]inary excretion of PGs observed by us and the similar results by Weber *et al.* (1977), [illegible]ould be in accordance with this hypothesis, which, however, must be regarded as tentative [illegible]nd awaits exploration in further experiments.

[illegible]his study [illegible]as supported by grants from the Swedish Medical Research Council (No. 2211) and from [illegible] Lillarefälldskapet. T. Devis[illegible] [illegible]as [illegible]siting scholar from S.F.R. Jugoslavia. Prostaglandins and [illegible] PGF2α were kindly supplied by Dr Udo Axén, The Upjohn Company, Kalamazoo, Michigan.

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TABLE III Excretion of immunoreactive $PGF_{2\alpha}$ ($iPGF_{2\alpha}$) and $iPGE_2$ in response to ... Food and water offered *ad libitum*.

Diet	Mean ± S.D $iPGF_{2\alpha}$ (μg/24 hrs)	Mean ± S.D $iPGE_2$ (μg/24 hrs)
(2.5 NaCl)	0.69±0.3	0.46±0.1
1 (0.–5 NaCl)	3.50±0.7	1.20±0.3
$p < 0.001$		

when rats were subjected to a low salt diet (0 06 ,o) and a depression of PGE_2 on a high salt diet (10) Their data are however open to alternative interpretations since it could be argued that the low levels seen during the high salt diet could be due to enhanced utilization of the endogenous precursors *in vivo* with a resulting low level of post mortem accumulation of PGE_2 in the kidney Conversely the high level of PGE seen post mortem in rats on a low salt diet could be due to low activity of the PG system *in vivo*

Our data collected in a different species and in a manner circumventing the difficulties discussed above nevertheless confirm and extend the observations of Tobian and O'Donnell (1976) that a decreased sodium intake is associated with an increased activity of the renal PG system or with a shift in arachidonic acid metabolism.

Weber *et al* (1977) have in a study independent of ours reported a similar inverse relationship between dietary sodium and urinary output of PGs in the rabbit with one important difference. These authors found a significant change only in the output of PGE_2, but not in $PGF_{2\alpha}$. They showed that the intrarenal levels of the PGE_2-9-ketoreductase was stimulated by the high salt intake and the authors suggested that the increased formation of PGF could play a role in regulating renin release In the present study clearcut and statistically highly significant changes were seen both in $PGF_{2\alpha}$ and PGE excretion rates. The increase in $PGF_{2\alpha}$ excretion was seen both when the analyses were performed with radioimmunoassay and with gas chromatography-mass spectrometry and can not be due to limitations of methodology The reason for this discrepancy can not be explained at present

In the first experiment, similar in design to that of Weber *et al* (1977), the animals were fed *ad libitum* It was noticed that these animals ate and drank differently on the high and low salt diets. It could be argued that the changes in urinary PG excretion could be due to different intake of other food constituents than sodium or due to differences in water excretion. The second experiment was better controlled since the intake of food was standardized (30 g/kg/day) and no significant changes were seen in water excretion The results from this experiment therefore provide further proof of an inverse relationship between urinary PG and sodium excretion A third possibility for changes in renal output of PGs, which are unrelated to sodium excretion could be changes in urinary pH PGs are week acids and their excretion could conceivably be influenced by nonionic diffusion. This possibility could however be excluded since similar urinary pH values were observed during both diets.

There are several alternatives to explain our results. One is that the increased circulating angiotensin II levels after renin release in the sodium restricted animals could stimulate intrarenal formation of PGs. Other studies with perfused rabbit kidney (Gagnon *et al*

Acta. Physiol. Scand. 1978. 103. 107–109

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Nicotine inhibits the release of 6 keto-prostaglandin $F_{1\alpha}$ from the isolated perfused rabbit heart

By

ÅKE WENNMALM

rostaglandin I (prostacyclin, PGI_2) is a newly discovered prostaglandin with potent vasodilator and platelet anti-aggregatory properties (Gryglewski *et al.* 1976, Johnson *et al.* 1976). rostacyclin is formed in vascular tissues from various organs and species (Gryglewski *et al.* 1976). The compound degrades spontaneously in aqueous solution to its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (Johnson *et al.* 1976). This was recently shown to be the major prostaglandin released from the perfused rabbit and rat heart (Isakson *et al.* 1977, De Deckere, Nugteren and Ten Hoor 1977), and it has been suggested that the compound protects the coronary circulation against the formation of blood platelet aggregates (De Deckere *et al.* 1977). Earlier studies in our laboratory demonstrated that the synthesis of PGE in the isolated rabbit heart is stimulated by nicotine (Wennmalm and Junstad 1976, Wennmalm 1977). We now report that nicotine inhibits the formation of PGI_2 in the rabbit heart, as reflected by the appearance of 6-keto-$PGF_{1\alpha}$ in the cardiac effluent.

10 isolated rabbit hearts were perfused according to Langendorff with heated and gassed Tyrode solution. ^{14}C-arachidonate (New England Nuclear, 5 μCi, sp act. 60 Ci/mole) was infused at a rate of 0.25 μCi/min during two 10 min periods separated by a 10 min interval. The cardiac effluent was collected during the infusion of isotope and for the following 5 min. Nicotine was added to the Tyrode solution after the end of the first effluent collection period to produce final concentrations of 3 · 10^{-} · 10^{-4} M. Perfusion with nicotine containing solution was maintained during the second isotope infusion and to the end of the collection of effluent. The effluents from the hearts were extracted twice at pH 3–3.5 with an equal amount of ethyl acetate. After evaporation, the residue was subjected to thin layer chromatography (TLC) using 0.25 mm DC Fertigplatten Kieselgel F 254 (Merck) in solvent ethyl acetate-acetic acid-2,2,4-trimethylpentane-water (90:20:50:100) (Hamberg and Samuelsson 1966), against standards of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2, PGA and Na-arachidonate. Radioscans of the chromatograms were developed, using a Berthold Dunnschicht-Scanner.

During the infusion of isotope 75 ± 3% (mean ± S.E., n = 10) of the radioactivity was retained in the organ. A major portion of the activity in the effluent (55 ± 6%) was extracted ethyl acetate. The radioactivity released during the perfusion with nicotine-free Tyrode

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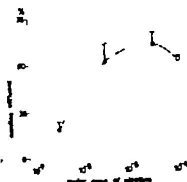


Fig. 2. Inhibitory effect of different concentrations of nicotine on the outflow of 6-keto-$PGF_{1\alpha}$ in the cardiac effluent. Ordinate: percent inhibition of the ratio between the amplitude of the 6-keto-$PGF_{1\alpha}$ peak and the mean amplitude of the PGE_2 and $PGF_{2\alpha}$ peaks, compared to controls. The control ratio, corresponding to 100 in the figure was 0.91 ± 0.08 (= 10). This obtained in effluents from hearts not treated with nicotine.

…elet anti-aggregatory PGI_2 may constitute a biochemical basis for the link between …bacco smoking and cardiovascular disease.

…is study was supported by the S…edish Tobacco Company and by the Swedish Medical Research Council, …ject 04X-4341. PG standards were kindly supplied by Dr J. Pike (Upjohn Company).

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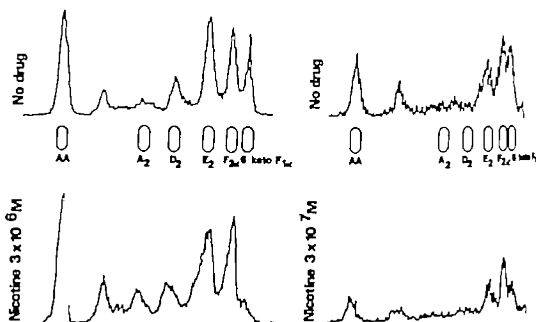
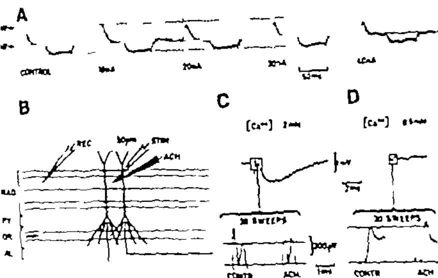


Fig. 1 *Upper panels* Radio-scans (thin-layer chromatography) of the ethyl acetate extracted cardiac effluent collected during infusion of ¹⁴C-arachidonate (0.25 μCi/min). No drug added to the perfusion solution. The radioactivity appears in three major peaks, corresponding to 6-keto-PGF1α, PGF2α, and PGE2. *Lower panels* Radio-scans of the cardiac effluents as above. Nicotine, at the concentrations indicated, was added to the perfusion solution 5 min before the infusion of isotope. Note that the peak corresponding to 6-keto-PGF1α in the lower left radio-scan is almost absent. In the lower right radio-scan, the 6-keto-PGF1α-peak is also depressed in relation to the PGF2α-peak in comparison with the control (upper right panel).

solution appeared in three major peaks, chromatographing in parallel to 6-keto-PGF1α, PGF2α, and PGE2. Sometimes there were also minor peaks in parallel to PGD and PGA (Fig. 1) The radiochromatograms from effluents collected during perfusion with nicotine containing Tyrode solution displayed a different pattern, inasmuch as the peak corresponding to 6-keto-PGF1α was depressed (Fig. 1) The inhibition of the 6-keto-PGF1α peak was dose-dependent, being about 60% at a nicotine concentration of 3 10⁻ M (Fig 2)

Nicotine is probably the most common of the drugs used by man The plasma levels of nicotine during cigarette smoking have been reported to range between 30 and 40 ng/ml (Armitage *et al* 1974) which corresponds to the lower dose used in the present perfusion experiments (3 10 M) Thus, the possibility that tobacco smoking affects the myocardial synthesis of PGI in man must be considered. It is well known that tobacco smoking subjects display increased morbidity in cardiovascular diseases The present observation that nicotine inhibits the myocardial formation of 6-keto-PGF1α, precursor of the vasodilating and



1 *A* The amplitude of EPSP was reduced when ACh was applied to the synaptically activated apical dendrites. Electrophoresis was performed with currents as indicated. The membrane resistance, measured with a hyperpolarizing current (0.4 nA, 50 ms) and the resting membrane potential did not change. Each trace is the average of 10 sweeps. *B*. Electrode arrangement used to test the excitability of afferent fibers. *C*. Upper trace: Presynaptic volley preceding the monitored EPSP. Lower trace: Highest amplification of the presynaptic volley. The amplitude was increased when ACh was applied near the stimulating electrode. Each trace is an average of 30 sweeps. *D*. As C after synaptic transmission was depressed by reduced Ca^{++} concentration.

reduced to 0.5 mM (Fig. 1 D) and thus the effect was not mediated by interneurons. Atropine abolished the effect of ACh independently of the concentration of Ca.

The experiments indicate that ACh may be a transmitter in the hippocampus of the rat both pre- and postsynaptically.

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Acta physiol. scand 1978, 103, 110–111
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Inhibition produced by iontophoretically applied acetylcholine in area CA1 of thin hippocampal slices from the rat

By

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Received 20 January 1978

Pyramidal cells in area CA1 of the hippocampus are excited by iontophoretically applied acetylcholine (ACh) This excitatory effect is blocked by atropin (Biscoe and Straughan 1966) Recent *in vitro* experiments suggest that the excitation is due to depolarization and is accompanied by an increase in membrane resistance (Dodd Dingledine and Kelly 1977)

I have investigated the effect of ACh in different regions of the dendritic tree in area CA1 of the transverse hippocampal slice preparation (Skrede and Westgaard 1971 Schwartzkroin 1975) The population spike evoked by an electrical stimulus in stratum radiatum was recorded in the pyramidal cell layer An ACh-containing glass pipette (resistance 50–80 MΩ) was used for iontophoretic application of ACh in discrete areas of the dendritic tree

An unexpected decrease in the amplitude of the population spike was produced when ACh was applied in areas where stimulated afferents made synaptic contacts. In other areas ACh enhanced the population spike or it had no effect Both effects of ACh were blocked when the experimental chamber was perfused with 10^{-6} M atropine added to the medium. In accordance with the report by Biscoe and Straughan (1966) pyramidal neurons, spontaneously active or driven iontophoretically by L-glutamate, were excited by ACh. It is thus unlikely that ACh produced spike inactivation as seen when L-glutamate is applied in large doses (Schwartzkroin 1975)

Intracellular recordings from pyramidal neurons showed that the depressing action of ACh was accompanied by a reduced amplitude of the evoked EPSP (Fig. 1 A) The resting membrane potential and membrane resistance were unchanged

To investigate the possibility that the site of action was presynaptic I measured the excit[illegible] ability of afferent fibers in the region of ACh application (Wall 1958 Eccles 1964). The diagram in Fig. 1 B shows the electrode arrangement. The stimulus and recording electrodes were placed in the stratum radiatum 400 μm apart, parallel to the afferent fiber direction A presynaptic volley was recorded as a negative–positive potential preceding the EPSP[illegible] from a group of nearby neurons (Fig 1 C) (Andersen *et al* 1977) When ACh was applied iontophoretically a few μm from the site of stimulation the amplitude of the presynaptic volley increased The increase was the same when the extracellular Ca[illegible] concentration was

Acta physiol. scand. 1978. 103. 113–119

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Effects of sodium salicylate on plasma insulin concentration and fatty acid turnover in dogs

By

Harald Vik-Mo, Knut Hove and Ole D. Mjøs

Received 13 June 1977

Abstract

Vik-Mo, H., K. Hove and O. D. Mjøs. *Effects of sodium salicylate on plasma insulin concentration and fatty acid turnover in dogs.* Acta physiol. scand. 1978. 103. 113–119.

The effects of intravenous sodium salicylate administration on plasma concentrations of insulin, free fatty acids (FFA) and glucose were studied in intact, unanesthetized dogs both during basal and norepinephrine stimulated lipolysis. In both situations sodium salicylate reduced the plasma concentrations of insulin. The reduction was associated with decreased plasma FFA concentrations and FFA turnover rate, while plasma glucose concentrations remained unaltered. The reduced plasma insulin concentrations effected by sodium salicylate are most likely secondary to the concomitant fall in plasma FFA concentrations due to inhibition of FFA mobilization from adipose tissue.

Key words: Fatty acids, fatty acid turnover, insulin, norepinephrine, sodium salicylate.

New interest in the mode of action of salicylates has been created by recent findings that acetylsalicylic acid (ASA) might reduce the incidence of acute myocardial infarction in man (Boston Collaborative Drug Surveillance Group 1974, Elwood *et al.* 1974), and that ASA (Moschos *et al.* 1975, Vik-Mo and Mjøs 1977) and sodium salicylate (SS) (Vik-Mo and Mjøs 1977) reduced the size of an acute myocardial ischemic injury in dogs. These beneficial effects have been related to the well known reduction of plasma free fatty acid (FFA) concentrations induced by salicylates (Carlson and Östman 1961, Bizzi, Garattini and Veneroni 1965).

The mechanism for the plasma FFA lowering effect of salicylates is not clear, however, and whether insulin is involved is not known. Although an antilipolytic effect of salicylates on adipose tissue has been demonstrated *in vitro* (Schönhöfer *et al.* 1973, Stone, Brown and Steele 1969), the plasma FFA lowering effect of salicylates *in vivo* can also be due to other mechanisms. Since insulin is an important regulator of lipolysis, an elevation of plasma insulin concentration by salicylates, as reported by Field, Boyle and Remer (1967) in man, would reduce FFA mobilization from adipose tissue. On the other hand, changes in plasma concentrations of insulin might be a consequence of alterations in plasma concen-

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trations of some of the substrates known to affect insulin regulation, i.e. glucose, amino acids, ketone bodies and FFA (Goodman 1974).

In the present study the effects of sodium salicylate infusion on plasma concentrations of insulin, glucose and FFA were investigated in order to establish possible relationships between the plasma FFA lowering effect of salicylates and the plasma insulin concentration. To obtain a wide range of FFA levels anesthetized dogs were given sodium salicylate before and during isoprenaline-stimulated lipolysis.

In addition to the possible effects of salicylates on lipolysis in adipose tissue the reduction of plasma FFA concentration induced by salicylates could be effected by an increase in tissue FFA extraction. Since salicylates *in vitro* have been reported to reduce fatty acid binding to albumin (Dawkins, McArthur and Smith 1970), a mechanism for the reduced plasma FFA concentrations might be a higher uptake of FFA in peripheral tissue at a given FFA concentration, as a consequence of lower albumin binding. A second aim of this study was therefore to examine if the plasma FFA lowering effect of sodium salicylate could alternatively be explained through a change in FFA turnover rate.

Methods

Animal preparation

Experiments were performed in 7 mongrel dogs of either sex, mean b.wt. 20 kg. After fasting overnight the dogs were anesthetized by an i.v. injection of sodium pentobarbital, 25 mg/kg b.wt. Supplementary doses were given at intervals when necessary. A femoral artery was cannulated for blood sampling and a femoral vein for infusion. Heparin, 100 i.u./kg b.wt., was given i.v. to avoid clotting of catheters.

Metabolic measurements

Arterial blood was collected into precooled heparinized plasma tubes. The blood was centrifuged immediately at 4 C, and the plasma was frozen and stored at − 20°C until analysed. All analyses were done in duplicate. Plasma glucose concentration was estimated by the glucose oxidase method (Boehringer Mannheim, Germany). Plasma insulin was measured by a radioimmunoassay (Poznanski and Poznanski 1969). Bovine insulin was used both as standard and for the production of anti-insulin antibodies. An antibody concentration giving maximal sensitivity in the range of insulin concentrations was chosen. Assays were performed to determine whether the presence of salicylate in the assay mixture alter the binding of labelled insulin to the antisera. Within the range of salicylate concentrations found in plasma no effect on binding was observed. The concentrations of FFA in plasma were determined by the titrimetric method of Dole (1956) as modified by Trout, Estes and Friedberg (1960). The presence of salicylate in plasma slightly influenced the FFA titration, thus 67 µmol/l per mmol/l of salicylate in plasma had to be subtracted from actually measured plasma FFA concentration.

Plasma salicylate concentration was assayed by a spectrophotometric method based on a colour reaction with ferric nitrate (Trinder 1954). Isolation of ^{3}H-palmitic acid from the plasma was performed in a two phase extraction system as described by Kaplan (1970). The radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer (Model 3320 Packard Instruments Co., Inc. Downers Grove, USA).

Experimental procedure

The effects of sodium salicylate infusion on arterial plasma concentrations of insulin, FFA and glucose during basal and isoprenaline stimulated lipolysis were studied in 7 dogs. The following experimental situations were studied in each dog:

1) Control situation I. Immediately after the blood sampling a continuous infusion of isoprenaline at a rate of 0.10–0.20 µg/kg min.

2) Isoprenaline situation. Blood sampling was performed 10 minutes after the start of isoprenaline infusion.

3) Control situation II 30 min after cessation of the isoprenaline infusion.

(4) Salicylate situation. Blood sampling was performed 30 min after slow i.v. injection of sodium salicylate 60 mg/kg b.w. freshly dissolved in saline and adjusted to pH 7.40, and followed by the continuous infusion of 0.15 mg/kg/min during the rest of the experiment.

(5) Isoprenaline-salicylate. Blood sampling was performed 10 min after start of isoprenaline infusion in the same dose as in 2).

In 4 of the 7 expts. the effects of sodium salicylate on FFA turnover during basal and isoprenaline stimulated lipolysis were studied. ^{3}H-palmitic acid with specific activity 300 μCi/μmol (The Radiochemical Centre, Amersham, England) was bound to 5% bovine albumin (No. A-6003, Sigma Chemical Comp. St. Louis, USA) in saline as described by Lassers, Kaijser and Carlson (1971). A continuous infusion of ... ml/min (0.5 μC/min) was given. The albumin-fatty acid infusion was started 90 min before the first control registration to give a constant ^{3}H-palmitic acid concentration. The radioactivity in the ^{3}H-palmitic acid infusate was determined in duplicate samples of the infusate.

Calculations

Specific activity of plasma FFA was calculated by dividing the radioactivity of each sample by the chemically measured FFA concentration.

The FFA turnover was determined from the equation

$$\frac{\text{radioactivity infusion rate (cpm/min)}}{\text{mean FFA specific activity (cpm/μmol)}}$$

μmol/min

Fractional turnover of FFA (per cent/min) was calculated as

$$\frac{\text{FFA turnover} \cdot 100}{\text{FFA pool}}$$

where FFA pool = FFA concentration × plasma volume. Plasma volume was assumed to be 5 per cent of the body weight.

Statistics

Wilcoxon non-parametric test for paired data (two-tailed) and Spearman rank correlation test were used to calculate probabilities (Snedecor and Cochran 1967). P > 0.05 was regarded as not statistically significant.

Results

The effects of sodium salicylate on arterial concentrations of insulin, FFA and glucose before and during isoprenaline-stimulated lipolysis are presented in Table I. Plasma insulin concentration increased from 0.82 ± 0.13 to 2.87 ± 0.49 ng/ml following isoprenaline infusion (mean ± S.E.M., P < 0.01). When sodium salicylate infusion was given significantly

TABLE I. Effect of sodium salicylate administration on arterial plasma concentrations of insulin, free fatty acids (FFA), glucose and salicylate before and during isoprenaline infusion in dogs. Mean ± S.E. of observations from 7 expts. are given.

Experimental situation	Insulin ng/ml	FFA μmol/l	Glucose mmol/l	Salicylate mmol/l
Control I (A)	0.82 ± 0.13	496 ± 98	6.46 ± 0.36	
Isoprenaline (B)	2.87 ± 0.49	2 157 ± 167	8.43 ± 0.78	
Control II (C)	0.63 ± 0.07	567 ± 113	6.23 ± 0.42	
Salicylate (D)	0.48 ± 0.06	411 ± 106	6.53 ± 0.27	1.19 ± 0.09
Isoprenaline-salicylate (E)	2.00 ± 0.30	1 439 ± 153	7.76 ± 0.65	1.08 ± 0.08
P_{A-C}		n.s.		
P_{C-D}	< 0.01	< 0.01	n.	
P_{B-E}	0.01	< 0.01	n.	

TABLE II Metabolism of free fatty acids (FFA) during sodium salicylate administration before and du[ring] isoprenaline infusion in dogs. Mean ± S.E. of observations from 4 expts. are given.

Experimental situation	Turnover rate of FFA µmol/min	Fractional turnover rate of FFA per cent/min
Control I	151 ± 52	31 ± 2
Isoprenaline	565 ± 88	31 ± 6
Control II	133 ± 40	35 ± 7
Salicylate	99 ± 19	37 ± 7
Isoprenaline-salicylate	352 ± 64	28 ± 5

lower plasma insulin concentrations were observed both before and during isoprenaline stimulated lipolysis ($P < 0.01$).

The average plasma fatty acid concentration was increased by isoprenaline from 496 ± [illegible] to 2 157 ± 167 µmol/l ($P < 0.01$). During sodium salicylate infusion lower FFA levels were observed both in the control situation and during isoprenaline-stimulated lipolysis (P [illegible]).

No significant differences in plasma glucose concentration could be detected between control situation I II and the salicylate situation (Table I), but the concentration was in[-] creased to a similar degree both in the isoprenaline and the isoprenaline-salicylate situation.

The plasma concentrations of salicylate were 1 19 ± 0.09 and 1 08 ± 0 08 mmol/l before and during isoprenaline-stimulated lipolysis respectively

A significant positive correlation was found between concentrations of plasma FFA and insulin ($r = 0.89$ $P < 0.01$ $n = 28$) and between glucose and insulin ($r = 0.66$ $P < 0.01$ n [illegible]) when data for basal and isoprenaline-stimulated lipolysis before and during salicylate ad[-] ministrations were pooled.

The turnover rate of FFA was reduced by sodium salicylate in the 4 expts. measured both before and during isoprenaline infusion (Table II) The fractional turnover rates of FFA were, however not changed by sodium salicylate.

Discussion

The main finding in the present investigation was reduced plasma insulin concentrations following sodium salicylate infusion both during basal and isoprenaline stimulated lipolysis. The insulin concentrations were positively correlated to the plasma concentrations of FFA and glucose. Since salicylate administration did not significantly influence the plasma glucose concentration either in the control situation or during isoprenaline infusion, it is unlikely that the observed effect of salicylate on insulin concentration could be mediated by a glucose action on the pancreas.

The dose of sodium salicylate in the present study was chosen so as to give a significant reduction of plasma FFA concentrations (Carlson and Østman 1961 Vik Mo and Mjøs 1976) Furthermore, the same dose of sodium salicylate or ASA effectively reduced the size of an acute myocardial ischemic injury in dogs (Vik Mo and Mjøs 1977) In man Carlson and Østman (1961) found a reduction of plasma FFA by 5 g of acety[l]s[alicy]l[ic acid] late giv[en]

healthy and diabetic subjects, and recently an oral administration of about g of sodium cylate effected a marked reduction of plasma FFA in healthy fasting students (V. I. Mo, ng and Mjos: to be published). Thus, the dose of salicylates given to obtain an effectie luction of plasma FFA is ordinarily somewhat higher than the dose given to humans for algesia, as also reflected in higher plasma concentrations of salicylates. Whether analgesic ses of salicylates might have some antilipolytic effect remains to be definitely established.

Our finding of reduced plasma insulin concentration after sodium salicylate infusion is in cordance with Arnold and Fernstrom (1976) who found a dose dependent sodium salicy te-induced reduction in insulin concentration in rats. In contrast, salicylates caused either slight elevation (Bellet *et al.* 1972, Field, Boyle and Remer 1967) or no change (Limbeck *al.* 1965) in serum insulin concentrations in humans. In these experiments, no attempt as undertaken to relate eventual changes in plasma insulin concentration to changes in asma fatty acid concentration.

It has been shown that fatty acids play a significant role in the regulation of insulin secre on. Infusions of FFA into the pancreatic artery in dogs gave rise to a marked elevation in mmunoreactive insulin in pancreatic enous blood (Crespin, Greenough and Steinberg 973). Furthermore, elevation of plasma FFA by *i.v.* infusion of long-chain fatty acids Greenough, Crespin and Steinberg 1967) or by infusion of triglyceride and heparin (Madison *t al.* 1968) increased the insulin concentration in dogs. In man, administration of nicotinic acid, potent antilipolytic agent, reduces insulin and FFA concentrations without changing lasma glucose concentration (Balasse and Ooms 1973). Since this drug has no direct inhibitory effect on pancreatic secretion of insulin (Malaisse, Malaisse Lagae and Mayhew 1967), the observed fall in insulin concentration has been explained through reduction in FFA concentration (Balasse and Ooms 1973). The possibility that sodium salicylate might influence pancreatic secretion of insulin independently of the changes in FFA concentration, should, however be considered. Coore and Randle (1964) observed reduced insulin secretion in fragments of rabbit pancreas incubated *in vitro* with glucose when salicylate was included in the incubation medium. Seltzer (1962), however found by cannulating the pancreaticoduodenal ein in dogs no change in the release of insulin activity after salicylate administration. Although the possibility of a direct effect of salicylates on insulin secretion in dogs cannot be ruled out, the correlation between changes in FFA and insulin concentration in the present investigation makes it reasonable to assume that the lowered plasma insulin concentration observed during sodium salicylate administration was, in fact, secondary to the reduction of plasma FFA concentration. Possible functional consequences of the lowered plasma insulin concentrations effected by sodium salicylate, remains, however to be determined.

Radio-labelled palmitic acid is suitable tracer for the study of total FFA turnover (Hagenfeldt 1975). In the present study we demonstrated that the FFA turnover rate was reduced by sodium salicylate. The fractional turnover rate, however was unchanged throughout the whole experiment, indicating that the reduced plasma FFA concentrations found after sodium salicylate administration was not due to increased uptake of FFA in the tissues. This observation is in accordance with previous study showing that the net uptake of FFA in the myocardium of dogs was unaffected by sodium salicylate infusion during basal lipo-

TABLE II Metabolism of *free fatty acids (FFA)* during sodium salicylate administration before and during isoprenaline infusion in dogs. Mean ± S.E. of observations from 4 expts. are given.

Experimental situation	Turnover rate of FFA µmol/min	Fractional turnover rate of FFA per cent/min
Control I	131±3	31±2
Isoprenaline	565±88	31±6
Control II	133±40	35±7
Salicylate	99±19	37±7
Isoprenaline-salicylate	35 ±64	28±5

lower plasma insulin concentrations were observed both before and during isoprenaline-stimulated lipolysis (P <0 01)

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Discussion

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lysis, and during isoprenaline-stimulated lipolysis net myocardial uptake of FFA were reduced by sodium salicylate (Vik Mo and Mjos 1976)

Since salicylates have been shown to inhibit prostaglandin synthesis in vitro (Vane 1971) the possibility that the plasma FFA lowering effect of sodium salicylate was mediated through prostaglandins warrants consideration. The potent prostaglandin synthesis inhibitor indomethacin (Vane 1971), however has no antilipolytic effect in vitro (Fredholm and Hedqvist 1975) and furthermore, prostaglandin PGE inhibits lipolysis induced by nerve stimulation in dogs (Fredholm and Rosell 1970) It is therefore unlikely that the plasma FFA lowering effect of sodium salicylate is mediated through prostaglandins.

Administration of heparin liberates the enzyme lipoprotein lipase to the circulation with hydrolysis of plasma triglycerides to FFA and heparin might therefore influence the lipid metabolism. In this study however we used overnight fasted dogs in which no significant effect of heparin on arterial FFA concentrations has been observed when comparable heparin doses were given (Frey 1967 Mjøs 1971)

In conclusion, intravenous sodium salicylate administration effected a reduction in plasma concentrations of FFA most likely through a non-insulin dependent inhibition of FFA mobilization. The reduced FFA mobilization by sodium salicylate seems to be due both to inhibition of adipose tissue lipolysis and to enhanced re-esterification of FFA within the same tissue (Vik Mo and Mjos 1978)

The skilled technical assistance of Thale Henden and Jan Raustandaksel is gratefully acknowledged. The work was supported by the Norwegian Council on Cardiovascular Diseases.

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ghosic neurons in other parts of the sympathetic nervous system. The reduction in the nber of noradrenaline (NA)-containing nerves during pregnancy—with an almost nplete disappearance near term followed by a restitution towards normal *post partum*—esents one drastic example of the special properties of the uterine sympathetic inner ion (Sjöberg 1968, Gårdmark, Owman and Sjöberg 1971, Owman *et al.* 1975). The nges ha e been followed by direct fluorescence microscopic visualization of neuronal 's in combination with chemical determinations of the total uterine level of the transmitter. The guinea-pig has for several reasons been chosen as a model for in estigations on the chanisms underlying these and related changes (Falck *et al.* 1974, Thorbert, Alm and nergren 1978), and the anatomy of the uterine autonomic innervation has therefore ently been described in detail in this mammal species (Thorbert *et al.* 1977). The present dy as undertaken to elucidate to which extent the fluorescence microscopic changes d alterations in the transmitter level in the uterus during pregnancy might invol e de erative phenomena, and their possible relationship to local factors associated with the wing conceptus. These factors can be particularly well studied in pregnant guinea pigs cause there are often fetuses only in one horn, lea ing the contralateral horn only slightly ger than the uterine horns of non-pregnant animals.

Materials and Methods

mals. The material consisted of guinea-pigs (nulli- or primiparous, respectively) of mixed strain and housed at various reproductive states. Nulliparous animals from the different periods of pregnancy were selected by palpation, and after killing under light ether anaesthesia the day of pregnancy as checked by measurement of uterus weight and crown-rump length of the fetuses (Draper 1920, Kaufmann 1969). The two groups were at 35–40 and 60–64 days of pregnancy. Animals from early pregnancy (18–23 days) were obtained by caging female animals, having a newly broken vaginal membrane together with male guinea-pigs for 3 days. The pregnant animals were killed 20 days later and the weight and crown-rump length of the fetuses were measured to obtain further check of the stage of pregnancy.

Primiparous animals were also studied at 1, 3, 6, and 12 weeks after delivery: the abdomen was opened under halothane (Fluothane, ICI) anaesthesia and aseptic conditions 2–5 days *post partum* to check whether pregnancy had been unilateral or bilateral. After closure of the abdomen, 10 mg of oxytetracycline (Terramycin, Pfizer) was given by a single i.m. injection.

Non-pregnant control animals in the dioestrous stage were used on day 7–12 of the oestrous cycle, determined as previously described (Thorbert *et al.* 1977).

Tissues. In non-pregnant and post partum animals, separate tissue preparations (Fig. 1) were taken from the last 2–4 mm of the uterus adjacent to the oviduct ("tubal end of the uterus"), the main part of the uterine horn, and from the cervix. The proprietary ligament, connecting the tubal end of the uterine horn with the lower ribs, was also dissected out. In early pregnancy (18–23 days post coitum) and in mid-pregnancy (35–40 days), tissues were obtained from the tubal end of the uterus and the cervix as above, but the main part of the uterus was separated into the following portions (Fig. 1): the uterine horn lacking fetuses and designated the "empty horn", the distended part of the fetus-containing horn, called the "peri-fetal uterus", and the non-distended part of the horn adjacent to (or between) the fetuses, designated the "para-fetal uterus". In pregnancy of longer duration than 45 days, such non-distended part of the uterus could no longer be distinguished (Fig. 1). Paracervical tissue from animals at late pregnancy was studied either as whole-mounts (Thorbert *et al.* 1977) or after freeze-drying and sectioning (see below).

Paracervical tissue from non-pregnant animals and at 60–64 days of pregnancy was frozen as above and sections (20 μm thickness) were prepared in a cryostat at −25°C, dried *in vacuo* in a desiccator over phosphorous pentoxide overnight, exposed to formaldehyde gas (see below) and further processed for fluorescence microscopy.

Uterine uptake of α-methyl-NA. 4 animals at 60–64 days of pregnancy received 1 mg/kg of the "false adrenergic transmitter" α-methyl-NA (Corbasil, Hoechst), by a single i.v. injection, and were killed 15 min

Acta physiol. scand. 1978 103. 120–131

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Regional changes in structural and functional integrity of myometrial adrenergic nerves in pregnant guinea-pig, and their relationship to the localization of the conceptus

By

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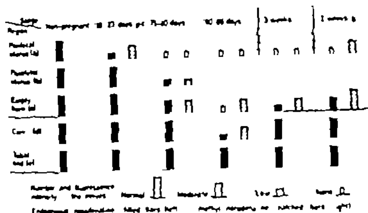
Received 6 October 1977

Abstract

THORBERT G P ALM CH OWMAN, N-O SJÖBERG and B SPORRONG *Regional changes in structural and functional integrity of myometrial adrenergic nerves in pregnant guinea-pig and their relationship to the localization of the conceptus* Acta physiol scand. 1978 103 120–131

Pregnancy is accompanied by reduction in uterine noradrenaline and the study was undertaken to investigate associated structural and functional integrity of the sympathetic nerves in the organ. The formaldehyde-induced fluorescence of adrenergic nerves was studied in different uterine regions before and after *in vitro* incubation or injection with α-methyl-noradrenaline in pregnant and puerperal guinea-pig uterus at 6 time periods, from early pregnancy (about 20 days *post coitum*) to 3 months *post partum*. The changes were related to the position of the fetuses, which were often present in only one of the two uterine horns. There was a drastic loss of fluorescent adrenergic nerves in myometrial tissue from horns distended by fetuses. Attempts to restore the fluorescence by incubation or injection with α-methyl-noradrenaline were essentially ineffective. Tissues from uterine regions outside (and not distended by) fetuses (in the case of early pregnancy), from horns devoid of fetuses (in the case of unilateral pregnancy), and from the cervix also lost their noradrenaline fluorescent nerves, but this occurred at a much later stage of pregnancy. After treatment with α-methyl noradrenaline a fluorescent plexus of sympathetic nerves could be restored to a considerable extent in these latter tissues. In puerperal animals the horn that had been devoid of fetuses regained its endogenous fluorescence much faster and the uptake of α-methyl-noradrenaline was more efficient, than in the horn which had contained fetuses. In this latter horn clear signs of restoration of endogenous adrenergic fluorescence and a clear uptake capacity was not found until 3 months after delivery. In the tubal end of the uterus, the reduction in the number of fluorescent nerves was only insignificant, and the region thus clearly differed from the rest of the uterus. It is concluded that (1) there are clear regional differences with regard to the disappearance of the noradrenaline transmitter in the uterus, (2) this disappearance in early pregnancy is related to the position of the conceptus, and (3) the changes involve degenerative and regenerative phenomena as well as alterations in transmitter levels of intact neurons.

The mammalian uterus receives a well-developed supply of adrenergic nerves (Owman, Sjöberg and Sjöstrand 1974) which innervate the myometrial smooth muscle cells as well as the uterine vascular bed (Silva 1967 Marshall 1970, Hervonen and Kanerva 1973 Sporrong *et al* 1977). These nerves differ both anatomically and functionally from the post-



2. Summary of changes in uterine adrenergic nerves in pregnant and puerperal guinea-pigs, based on fluorescence microscopic analysis described in the text. The uterine regions refer to those illustrated in 1, and the stages are expressed in terms of time post coitum (p.c.) and post partum (p.p.). The tables not take into account quantitative regional differences in the innervation (cf. Thorbert et al. 1977) but changes in reference to the non-pregnant, dioestrous control animal. The filled bars represent the number fluorescence intensity of nerves containing endogenous noradrenaline, whereas the hatched bars [illegible] the total number of green-fluorescent nerves present after incubation or injection with α-methyl-noradrenaline. It should be noted that this treatment sometimes results in nerve fluorescence intensity [illegible] exceeds that of the noradrenaline fluorescence found in the non-pregnant controls. Incubation [illegible] have usually been performed only when there was a reduced number of noradrenaline-fluorescent nerves in [illegible] given tissue. As can be seen from Fig. 1, a perifetal part of the uterus is not distinguishable [illegible] and beyond late pregnancy the uterine horn then consists only of perifetal tissue (cf. Fig. 1 C).

other nerves, varicosities appeared somewhat swollen and the segment between the individual varicosities had little or no fluorescence. Still other fibres were not distinctly defined, suggesting of a diffusion of the fluorophore. In two animals studied at the end of this stage of pregnancy there were no fluorescent terminals visible at all in the perifetal uterus. Incubation of tissue slices from the perifetal uterus induced a fluorescence in some nerves, but the nerve plexus was very scarce in comparison with uterine tissue from non-pregnant animals.

In the remainder of the uterine horn (between fetuses and in the tubal end), as well as in the uterine horns lacking fetuses, no certain changes could be observed in the adrenergic innervation except for a tendency to a general increase in fluorescence intensity of the nerves.

Mid pregnancy (35–40 days *p.c.*). At this stage the perifetal uterus was completely devoid of fluorescent nerves (Fig. 3 A), and in the parafetal uterus a general moderate decrease in the number as well as the intensity of the fluorescent nerves had occurred (Fig. 3 B). The uterine horn in these latter regions was not subject to any distension, since they were selected from uteri containing only one or two fetuses.

In the main part of the empty horn the picture of the innervation was more variable, ranging from a pattern seen in non-pregnant animals to a clear decrease in nerve fluorescence, though not to the extent seen in the parafetal uterine tissue. In the tubal ends of the uterine horns whether containing fetuses or not, and in the cervix, little or no alterations could be

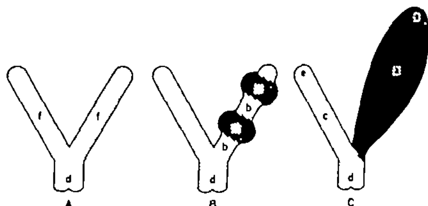


Fig. 1 Schematic representation of the guinea pig uterus showing the various regions from which the preparations were taken. A, non-pregnant and *post partum* animals B, early and mid pregnancy C late pregnancy: a, perifetal uterus b, parafetal uterus, c, empty horn d cervix; e, tubal end of the uterine f main part of the uterine horn.

later for dissection of uterine tissue. The uptake of α-methyl-NA *in vitro* was studied according to the procedure described by Hamberger (1967) in preparations from 3–5 animals at the other stages. Minced tissue slices from the main part of the uterine horns of all types (Fig. 1) were incubated for 10 min in Krebs Ringer bicarbonate buffer containing 0.1 1 or 10 μg/ml of the amine Immediately after completed incubation, the slices were frozen in a propane/propylene mixture at the temperature of liquid nitrogen, and further processed for fluorescence histochemistry (see below). Uterine tissues from 4 non-pregnant animals treated with 0.5 mg/kg reserpine (Serpasil, C ba) i p 24 hours beforehand (which completely abolished fluorescence from all adrenergic nerves), were also incubated in the presence of α-methyl-NA as a further check t ascertain that a neuronal uptak did occur under the conditions used.

Histochemical procedure Tissue pieces from the various uterine regions (Fig. 1) as well as the α-methyl-NA-incubated preparations were processed accordi g to the fluorescence technique of Falck and Hillarp (see Björklund Falck nd Owman 197?) F rmaldehyde of fixed humidity (70%) was used in order to allow f more accurate comparison of changes in fluorescence intensity With the formaldehyde technique, the fluorophore f α methyl-NA has the same spectral characteristics as that of NA, and the fluorescence yield is equal (Björklund *et al* 1972)

Results

The organization of the uterine adrenergic innervation in non-pregnant guinea-pigs has recently been described in detail (Thorbert *et al* 1977)

Incubation of the uterine tissue from reserpine treated non-pregnant animals (to check the reliability of the incubation technique) produced a near full restitution in the number and fluoresence intensity of the adrenergic nerves, though the axons had a more smooth (less varicosed) appearance than in preparations from non-reserpinized animals.

Changes in the histofluorescence of the adrenergic nerves in the various uterine regions at the different stages of pregnancy and *post partum* have been compiled schematically in Fig. 2.

Early pregnancy (18–23 days p c.). Tissue pieces taken from the perifetal uterus showed clear fluorescence microscopic changes. In most animals, the number and fluorescence intensity of the adrenergic nerves were markedly reduced, with additional changes also in the microscopic appearance of the individual axon terminals. Sometimes the delicate fibres did not show any well-defined varicosities characteristic of the normal adrenergic nerve terminals

\ most instances the main part of the empty horn showed the same picture as the :ralateral (fetus-containing) horn, though the absence of adrenergic nerves was less obvious in the former The tubal end of the empty horn exhibited an almost unchanged energic innervation compared to non-pregnant control animals.

n parametrial tissue adjacent to the uterine horns, i.e. the peritoneal membrane con-ing the primary ramifications of the uterine artery (cf Thorbert et al 1977), the adrenergic ve terminals displayed clear reduction in fluorescence intensity and in the number per 3 of tissue.

The clusters of ganglion cells in the paracervical fibro-adipose tissue could regularly be h. The general arrangement of these ganglion formations and of the individual ganglion s was appreciably the same as in non-pregnant animals, though the axon bundles leaving ganglia often tended to show an increased fluorescence intensity. Perivascular fluorescent e terminals were somewhat more abundant and intense than in the parametrial tissue cribed above.

njection of α-methyl-NA or incubation in the presence of the amine produced principally same results in all of the animals studied during late pregnancy. In the main part of us-bearing horns, no uptake into nerve-like structures could be observed and the tissue hence completely devoid of fluorescent nerves, whether treated with α-methyl-NA or t. However in the empty horn, a small to moderate increase in both the number and fluo-scence intensity of the adrenergic nerves occurred under the in vivo and in vitro conditions. similar degree of increase was also seen in the cervix after injection of α-methyl-NA.

Post partum 1 and 3 week. In the cervix and the tubal ends of the uterine horns, a recovery the number and fluorescence intensity of the adrenergic nerves could be recognized already eek post partum, and complete normalization was attained within the third week. owever, the main part of the horn that had contained fetuses showed no fluorescent erves at 1 or 3 weeks after parturition. In the main part of the empty horn a clearcut turn of nerve fluorescence, having an intensity resembling that of non-pregnant control nimals, could be distinguished, though the number of fibres was small and variable. haracteristically some of the re-appearing fluorescent nerves differed from those seen in ne uterus of the non-pregnant control animals in that many of them had a thicker and moother appearance, and a high fluorescence intensity. The picture is interpreted as ransmitter accumulation in the preterminal portion of the axons. Other fibres were more elicate than axon terminals in general, with varicosities of varying size and interspersed at rregular intervals.

Incubation with α-methyl-NA in vitro did not produce any appearance of fluorescent nerves in tissue slices from the main part of uterine horns that had contained fetuses. In the empty horn the number of fluorescent nerves seen after incubation was moderately or sometimes even markedly increased compared to corresponding non-incubated tissue. However the density of innervation was still somewhat lower than in the uterus from non-pregnant controls.

Post partum 6 and 12 weeks. In the main part of uterine horns that had contained fetuses, the fluorescence microscopic picture was not noticeably different from that of the groups analysed at 1–3 weeks post partum: thus no or very few fluorescent nerves were visible. In



Fig. 3 Fluorescence photomicrograph of sections from a fetus-containing uterine horn of an animal a days of pregnancy (A) Fluorescence microscopic appearance of the perifetal (distended) part of the ut horn. (B) Parafetal (non-distended) part of the uterine horn. That part of the uterus surrounding the (A) is completely devoid of adrenergic nerves, whereas in the non-distended part from the same horn, between the fetuses (B), the fluorescent nerves resemble those found in non-pregnant animals, their umber is only slightly reduced. Subtle differences in comparison with the innervation of the pregnant uterus incl de swollen varicosities and sometimes disappearance of the intervaricose fluorescence. × 120.

detected in the number of nerves, and the fluoresence was unchanged or possibly ev increased

Incubation with α-methyl-NA *in vitro* of uterine tissue from perifetal, parafetal, or fr empty horns produced no clear changes in the number of fluorescent nerves compared non-incubated tissue. However when nerves did occur their fluorescence intensity increased after incubation with the amine.

Late pregnancy In the main part of fetus-bearing uterine horns there was a uniform total lack of adrenergic nerves, irrespective of whether the tissue specimens were taken fro the uterine wall underneath or far away from the placenta A very small number nerves with low fluorescence intensity persisted in the cervix, most often with relations to blood vessels.

In the tubal end of the uterine horn and in the suspensory ligament, however the fluorescence microscopic picture was clearly different, so that a great number of vascular and non-vascular nerves with a moderate florescence intensity still persisted. Nevertheless, the region contained a somewhat smaller number of nerves with a lower fluorescence intensity compared with non-pregnant control animals In some preparations of the suspensory ligament there was a definite increase in the fluorescence intensity of the preterminal fibres located among the axon terminals having a reduced fluorescence intensity Within the suspensory ligament, topographic differences were observed in the appearance of the nerve terminals In the cranial parts of the ligament the fluorescence microscopic appearance was normal the axons being equipped with distinct varicosities having a well-defined outline (*i.e* without any tendency to diffusion of the fluorophore) In the caudal parts, as in the tubal end of the uterine horn, the nerve terminals had a smooth appearance. Their fluorescence intensity was lowered and they had a more diffuse contour

as the non-pregnant weight). During early pregnancy the NA fluorescence was unchanged if anything, increased in this horn followed by a progressive reduction, so that no or few nerves remained visible (with a reduced intensity) at parturition and one week *partum*. Thereafter the innervation was greatly restored. Incubation in the presence the α-methylated NA analogue resulted in a fluorescence microscopic picture indicating at least the majority of adrenergic nerves had retained a good capacity for catecholamine ake and accumulation. This suggests that during pregnancy the structure of the energic nerve network in the empty horn has to a large extent remained intact, and that nerves have lost their NA mainly as a consequence of some humoral (local or systemic) or affecting mechanisms, such as transmitter synthesis, retention, release etc.

Parafetal uterus designates that part of the uterine horn which is located adjacent to or een fetuses, and which can be distinguished as an essentially non-distended part up to proximately 45 days of pregnancy (depending on the number of fetuses present). During time such regions could be separated from the rest of the fetus-containing horn, the orescence microscopic picture of the innervation followed the same pattern of changes that observed in the empty horn. There is reason to believe that they reflected altered NA els in an adrenergic nerve plexus where the majority—if not all—of the axons is intact.

Perifetal uterus distinguishes the uterine wall surrounding the entire conceptus, including placenta. Due to the increased space occupied by the fetus in advanced pregnancy -45 days), the perifetal uterus will later include almost the entire uterine horn except ends bordering the oviduct and cervix, respectively. Already at days 18–23 of pregnancy re was a marked reduction in the number and fluorescence intensity of adrenergic nerves the perifetal uterus, and from 35–40 days onwards all NA fluorescence was abolished and mained so during all subsequent time intervals studied. In no instance (except for a slight take during early pregnancy) did incubation with α-methyl-NA induce a reappearance of orescent nerve fibres in the perifetal myometrial slices, showing that some kind of nctional change has occurred, reflected in, e.g. a reduced axonal uptake of amine. In view previous findings of histological signs of nerve degeneration in the fetus-containing horn arderstrark *et al.* 1971) it is possible that these altered properties could be due to a structural mage of the nerves.

Tubal end of the uterine horn, whether containing fetuses or not, showed a tendency to increased nerve fluorescence during early and mid pregnancy. Near term no clearcut crease was observed in that of the empty horn, whereas the fetus-containing horn possibly owed slight reduction in the number and intensity of the adrenergic nerves. It could t be clarified, however to which extent the fluorescence microscopic picture reflected a ue decrease in the number of nerves or a dilution effect when the nerves had more attered localization in the enlarged organ. A normal fluorescence microscopic picture as seen during the *post partum* stages analyzed. Hence, the adrenergic nerves in this small gion of the uterus reacted different from those in the remainder of the organ. The tubal nd is a particularly well innervated part of the uterine horns receiving all of its adrenergic nervation via the suspensory ligament. This is a smooth muscle containing structure onnecting the lower rib and the cranial end of the uterine horn and it contributes with as much as 35% of the total NA content in the horn (Thorbert *et al.* 1977). Thus, the adrenergic

contrast to this, incubation in the presence of α-methyl NA produced a picture de- different from that seen at full term and during the first weeks *post partum* (when no fluorescence could be induced) At 6 weeks *post partum* a moderate number of fluorescent nerves was seen, and at 12 weeks the density of fibres was seemingly further increased in the myometrium and around blood vessels.

The endogenous nerve fluorescence in the main part of the uterine horn that had been devoid of fetuses during pregnancy showed a high degree of recovery although a complete restoration in the number of nerves had not yet been attained at 6–12 weeks *post partum* However a nerve plexus of essentially the same density as in the non-pregnant animals was achieved after incubation of tissue slices from these horns in the presence of α methyl NA

Discussion

The level of sympathetic neurotransmitter NA in the uterus can be influenced by sex steroids (Owman and Sjöberg 1977), not only secondarily as a result of the hormone-induced changes in organ weight, but also independently in as much as the nerves seem to constitute a separate target for the action of sex steroids (Broberg *et al* 1974 Thorbert *et al* 1976 Induction of pseudopregnancy in rabbit is followed by a reduction in uterine NA which can be related to increased progesterone levels in both tissue and plasma (Thorbert *et al* 1976) and reduced estradiol in plasma (Batra *et al* 1977) Further under the influence of estrogenic stimulation the neuronal content of NA in the uterus increases, an effect that can be counteracted by progesterone (Falck *et al* 1969) Also in guinea-pigs the adrenergic neurotransmitter can be changed in the uterus by sex steroids, and it has been found that combined estrogen and progesterone treatment decreases the total uterine content of NA (Thorbert *et al* 1978) In order to relate these changes—occurring only in the adrenergic system supplying the reproductive (and perhaps also lower urinary) tract—to more physiological situation, the guinea-pig estrous cycle (Thorbert *et al* 1978) and pregnancy has been examined separately (Owman *et al* 1975) It was shown that diestrous animals had higher uterine NA than estrous ones, and it was also found that the neutrotransmitter was almost entirely lost from the uterine sympathetic nerves at the end of pregnancy In view of the above mentioned observations, together with the finding that a fall in uterine NA during pregnancy took place also in uterine horns lacking fetuses, it has been suggested that humoral factors play a role in the disappearance of uterine neurotransmitter during pregnancy (Gårdmark *et al* 1971 Owman *et al* 1975) Such factors might either be of systemic nature or local related to the fetus and/or placenta.

The present study has revealed a complex sequency of changes in the uterine adrenergic innervation, related not only to various stages of pregnancy but also to different regions of the uterus and the location of the conceptus. The alterations will therefore be discussed and interpreted as they have been visualized histochemically in the separate anatomical regions (Fig. 1 and 2)

Empty horn The guinea-pig is particularly suitable for this kind of study since fetuses are often present in only one of the horns during pregnancy The contralateral horn essentially retains its non-pregnant appearance and the increase in weight is relatively modest (

is possible that the mechanical strain and excessive hypertrophy of the uterine wall ...ated with the growing conceptus are directly responsible for this nerve degeneration. ...ever also other factors seem to have been involved because loss of NA, combined ... a fading uptake mechanism, is seen also in earlier stages of pregnancy when the con... ...s is still small. The same holds true for the empty horns, where the myometrial hyper...ty is relatively small and mechanical strain is insignificant. Such other factors may ...de acceleration or adaptive changes in those normal degenerative and regenerative ...esses, which have been claimed to maintain the peripheral nerve network in a state of ...nous turnover (Barker and Ip 1966, Bjerre *et al.* 1975). They may also include more ...fic hormonal changes directly related to the situation of pregnancy. The finding of a ...rescence accumulation in otherwise only moderately fluorescent bundles of preterminal ...es arising in the paracervical ganglia and running in the suspensory ligament, ...ther with the swollen and distorted appearance of nerves in the myometrium, favours ...iew that a terminal nerve degeneration and regeneration take place in the uterus ...ore, Björklund and Stenevi 1973)).

...lso the horns devoid of fetuses during (unilateral) pregnancy showed a dramatic reduc... in nerv... fluorescence, so that no or only few adrenergic nerves remained visible at the ... of pregnancy in accordance with the course of disappearance of NA in the empty ...ine horns (Owman *et al.* 1975). The rate and pattern of disappearance of fluorescent ...ves differed from those in the fetus-containing horns (Owman *et al.* 1975), and by ...ubation with α-methyl-NA, the fluorescence could always be restored in a large number ... the nerves in the empty horns. This shows that in the latter horns most of the nerves, ...ough they had lost their endogenous NA, still retained a capacity for catecholamine ...take and accumulation. It is suggested that the transmitter in the empty horns had mainly ...appeared from a system of structurally intact nerves, whereas in the fetus-containing ...rns it ...as lost as a consequence of severe nerve damage and degeneration. Recent ...mical determinations of uterine tyrosine hydroxylase during pregnancy have shown that ... enzyme activity is reduced to ...ery low values (unpublished observations) in pace with ... disappearance of NA from the empty horn (Owman *et al.* 1975). Since this enzyme is ...e-limiting in the formation of NA, impaired synthesis may be a major cause of the NA ...uction in the empty horn during pregnancy. However, incubations in the presence of ...α-methylated NA analogue did not restore an entirely normal density of the nerve ...tern, which could mean that also in the empty horn nerves were damaged or degenerated, ...ough to ... much less extent than in the fetus-containing horn.

Since the empty horn undergoes relatively small changes in size, compared with the ...tensive enlargement of the fetus-containing horn, humoral rather than mechanical factors ...em to be responsible for the reduction in the formation and accumulation of transmitter ... the adrenergic nerve plexus of the empty horn. By analogy, it is conceivable that the ...ame mechanisms underlie the NA reduction in the non-distended parts of the fetus-con...taining horns (... parafetal uterus") during the earlier stages of pregnancy, when the ...nceptus is still small and only partially occupies the total uterine lumen. The same ...asoning also holds for the cervix region (which is common for both uterine horns), in ...hich the pattern of changes in the sympathetic innervation both histochemically and ...

fibres do not originate in the paracervical ganglia, nor are they affected by transection of hypogastric nerves, in contrast to the remainder of the uterine horns (Thorbert *et al* 19

Cervix The general pattern of changes in the nerve fluorescence resembled that for in the empty uterine horn However the fluorescence was not as markedly reduced at pregnancy and normalization after delivery was more rapid compared with the em horn.

Considerations on the mechanisms of NA disappearance The introduction of the Falck Hillarp histofluorescence technique (for references, see Björklund *et al* 1977) has allowed a detailed mapping of the distribution of the postganglionic adrenergic neuron system variety of sympathetically innervated tissues and organs from numerous species. It i general impression from this highly sensitive technique that the nerve plexus formed in the various locations have a characteristic and constant arrangement and that t transmitter is maintained at a fairly steady level in the individual neurons within the r of physiological changes in nerve activity (Burnstock and Costa 1975). This is reflected r rather constant NA concentration in a given sympathetically innervated organ. It is m only in situations of extreme experimental conditions (*e.g* Dahlström and Zetterst 1965 Nielsen and Owman 1967) or pharmacological interference with transmitter r and turnover (*e g* Malmfors 1965) that changes in endogenous NA level can be dete histochemically or chemically The female reproductive tract appears to be unique in t sense that fluctuations in the transmitter levels can be revealed during physiological c ditions such as the sexual cycle (Sjöberg *et al* 1977 Thorbert *et al* 1978). Such fluctua in the transmitter content of the nerves can be mimicked by exogenous hormone treatm (Owman and Sjoberg 1977) The extremes in the physiological variability of the adrener innervation in the female reproductive tract are seen during the end of pregnancy when t uterus may be almost devoid of noradrenaline-containing nerves (Sjöberg 1967) T detailed changes in the NA transmitter during pregnancy as recorded by fluorescence his chemistry in the present study are in good agreement with previous chemical determina tions of NA in the guinea pig uterus at various stages of pregnancy (Owman *et al* 19

Silver impregnation of tissue preparations taken from fetus-containing uterine horns late pregnancy in guinea pigs showed a reduced number of nerve fibres (compared with em horns), and several of the nerves showed signs of degeneration with a swollen appearan or were granulated and had disintegrated into small fragments (Gårdmark *et al*. 197 It is thus possible that the disappearance of NA-containing nerves from the fetus-contain horns is at *least partly due simply to loss of nerve structure This would agree* with t inability to regain nerve fluorescence after incubation in the presence of α methyl-NA. control experiments with reserpine in which the NA had been depleted from the nerv (of non-pregnant uterine tissue), a near complete restoration of the number of vis fluorescent nerves was obtained, showing that the incubating technique adequately demo strated the presence of NA-deficient nerves with a functioning axonal membrane pu There is evidence from silver impregnation studies (Gårdmark *et al* 1971) that the fet containing horn at late pregnancy also contains structurally more intact nerve fibres, b apparently with a functional damage as reflected in the defective uptake capacity It can assumed that this severe impairment represents one stage in a degeneration process.

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chemically (Owman *et al* 1975) followed those in the empty horn of the uterus (and in parafetal uterus) This humoral influence would be in accordance with the sensitivity of uterine adrenergic nerves to the action of sex steroids as evidenced by reduction in the NA levels during HCG induced pseudopregnancy (Thorbert *et al* 1976), oophorectomy (Fa... *et al* 1974) or treatment with estrogen and progesterone (Falck *et al* 1969 Thorbert 1978)

The fluorescent nerve plexus in the uterine horn that had been devoid of fetuses was restored to a great extent within three weeks after delivery whereas no endogenous NA fluorescence was visible in the other horns Even after twelve weeks only a small number of nerves were visible in the latter horns, though a nerve fluorescence could be induced by incubation with α methyl NA. It is assumed that there is a "refilling" of NA in the nerves of the empty horns, wheres the late reappearance of adrenergic fibres in the horns that contained fetuses mainly involves a regenerative phenomenon not until approximately twelve weeks *post partum* is a nerve plexus restored, but it still lacks the capacity required to accumulate sufficient amounts of endogenous NA for fluorescence histochemical visualization The time-course of reappearance of these nerves is in good agreement with that after treatment with 6-hydroxydopamine or antibodies to Nerve Growth Factor (Bjerre *et al* 1975) It should be mentioned that previously reported figures on the reappearance of uterine NA after delivery (Gårdmark *et al* 1971) are based on determinations which included also the cervix (and the tubal end of the uterine horn) where the NA restoration is more rapid. The NA values given are therefore higher than would be anticipated from the present fluorescence histochemical analysis of the main part of the uterine horns during the *post partum* period.

From earlier studies and the present fluorescence histochemical findings on the changes in the neuronal NA and the capacity of the uterine adrenergic nerves to accumulate exogenous transmitter analogue it can be concluded (a) that there are clear regional differences in the disappearance of uterine NA (b) that this disappearance is related to position of the conceptus, and (c) that the changes involve de and regeneration of nerves together with alterations in the NA level of intact nerve fibres.

This work was supported by gra ts from the Ford Foundation (680-0383A) and the Swedish Medical Research Council (04X 56).

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Tree types of motor units with different functional properties have been identified in at inferior oblique muscle (Lennerstrand 1974, 1975). The units with the fastest con-tions are probably composed of singly innervated fibres and the slowest units of multiply vated fibres of the slow amphibian type. The units of intermediate speed of contraction been suggested to consist of multiply innervated fibres resembling the slow fibres in muscle (Bach-y-Rita and Ito 1966, Lennerstrand 1974, 1975, Lennerstrand and Bach-y 1974, Bach-y-Rita 1975). It is not known how the properties of eye muscle fibres in that change from birth to the adult stage. Matyushkin (1967) reported that the contraction eye muscles in young rabbits were slower than those of adult muscle, and similar observa-s have been made on rat eye muscle by Close (1975).

recent years the postnatal development of skeletal muscle in the cat has been extensively died, particularly the functional differentiation of the fast and slow twitch fibre systems indlimb muscles (Buller, Eccles and Eccles 1960, Buller and Lewis 1965, Close and Hoh 7, Mann and Salafsky 1970, Bagust, Lewis and Westerman 1973, Westerman *et al.* 1973 mmarberg and Kellerth 1975 a, b). At birth all hindlimb muscles contract slowly. They to be composed of the same types of fibres (Nyström 1968 b, Maier and Eldred 1974, mmarberg 1974). During the first postnatal weeks a differentiation takes place and the muscles like the gastrocnemius increase their speed of contraction with age, while ers like the soleus remain slow throughout development (Buller *et al.* 1960, Buller and is 1965, Nyström 1968 a, Westerman *et al.* 1973). The differentiation of fast and slow traction seems to occur in parallel with changes in fibre composition (Nyström 1968 b, aier and Eldred 1974, Hammarberg 1974).

The purpose of the present investigation has been to study the postnatal development of extraocular muscle with physiological and histochemical techniques. A comparison with e development of fast and slow fibre systems of other cat muscles will be attempted al-though this will be somewhat difficult due to the lack of information on the postnatal deve pment of muscle fibres with multiple innervation, even in amphibian and avian muscle. ometric contractions have been recorded in the inferior oblique muscle of kittens of dif rent age and in the adult cat. It has not been possible to record from individual fibres or otor units, and conclusions have to be drawn from data obtained from the whole muscle. this paper the postnatal changes in twitch and tetanic contraction will be evaluated. In a bsequent paper the changes with age in fatigue properties and other post-tetanic effects ill be examined (Lennerstrand and Hanson 1978). The developmental changes in the histo-hemical fibre spectrum of the inferior oblique muscle will be described separately (Hanson, Nichols and Lennerstrand 1978). Preliminary reports on the results have been published lsewhere (Hanson, Lennerstrand and Nichols 1977, Lennerstrand, Hanson and Nichols 1977).

Methods

25 kittens and 3 adult cats were used in the study. They were arranged in 8 groups according to postnatal age. The number of animals in each group and their body weight are shown in Table I. I most animals muscles of both eyes were used for experiments.

The animals were anesthetized with pentobarbital (Nembutal®) 40 mg/kg b.w. injected intraperitoneally and the trachea and one femoral vein were cannulated. Additional doses of anesthetic were given [illegible] needed. The head was rigidly clamped with earpins and bite bar. The inferior oblique, the lateral and

Acta physiol. scand. 1978. 103. 132–143

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The postnatal development of the inferior oblique muscle of the cat

I. Isometric twitch and tetanic properties

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Received 28 November 1977

Abstract

LENNERSTRAND, G. and HANSON, J. *The postnatal development of the inferior oblique muscle of the cat. I. Isometric twitch and tetanic properties.* Acta physiol. scand. 1978. 103. 132–143.

The postnatal development of the inferior oblique muscle in the cat has been studied with physiological and histochemical techniques. This paper describes the changes with age in isometric twitch and tetanic response characteristics. The twitch amplitude increased and the twitch contraction time (*ct*) and half-relaxation time (*hrt*) decreased almost linearly from birth to adulthood. The relation between the rate of nerve stimulation and twitch *ct* and *hrt* changed during development with a threshold [illegible] appearing at 10 weeks. Twitch responses in cats 6 weeks of age or older were of longer duration than in younger cats, in spite of the longer *ct* and *hrt* in young cats. Fusion frequency of the tetanic responses [illegible] a constant level in muscles 6 weeks or older. The maximum rate of tension rise remained the same from birth to 6 weeks of age and later increased markedly up to 20 weeks of age. The contracture induced by succinylcholine was the same in muscles of all ages. These data were related to previous findings on the postnatal development of fast and slow muscles and motor units in the hindlimb of the cat. A difference of the development of fast and slow eye muscle fibres is suggested. Slow fibres seemed to have completed their maturation at about ten weeks of age, while the development of fast fibre properties continued, presumably up to the adult stage.

Key words: Postnatal development, cat extraocular muscle, fast and slow fibres, contractile properties, twitch and tetanus

With regard to muscle fibre structure, adult eye muscle is known to differ markedly from other muscles. The inferior oblique muscle of the cat has been shown to contain singly (focally) innervated muscle fibres of the same types that exist in mammalian skeletal muscle (Alvarado and van Horn 1975), but in addition multiply innervated fibres, not seen in ordinary skeletal muscle, have been identified (Hess and Pilar 1963, Peachey, Takeichi and Nag 1975, Alvarado and van Horn 1975, Mayr 1975). The multiply innervated fibres are of 2 types, one of them resembling slow fibres in amphibian muscle and the other slow fibres in avian muscle (Peachey 1971, Peachey *et al.* 1975, Alvarado and van Horn 1975, Mayr 1975).

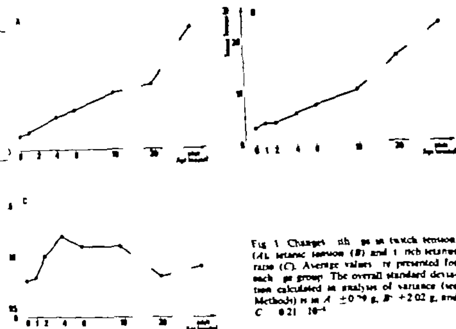


Fig. 1. Changes with age in twitch tension (A), tetanic tension (B) and twitch/tetanus ratio (C). Average values are presented for each age group. The overall standard deviation calculated in analysis of variance (see Methods) is in A ±0.79 g, B ±2.02 g, and C 0.21 10^{-1}

tanus tension increased with age in about the same manner. At all ages the maximum tetanic tension was obtained at stimulus rates that produced a smooth tension curve, i.e. the fusion frequency. This is well known from previous studies of adult cat eye muscle (Barmack, Bell and Rance 1971; Lennerstrand 1974), and seems to hold also for developing eye muscles.

Twitch/tetanus ratio. The mean twitch/tetanus ratios for each age group are shown in Fig. 1 C. The values were low at birth. They shifted to a higher level in animals between [illegible] and 10 weeks of age but became low again in the adult cat. These age variations were statistically significant at the 5 % level.

Twitch contraction. Contraction times (*ct*) and half-relaxation times (*hrt*) of the twitch have been plotted against age in Fig. 2 A and B. For comparison, mean values of the same parameters during development of the gastrocnemius and soleus muscles, obtained from the work by Hammarberg and Kellerth (1975 a), have been included in the graphs. Disregarding the fact that the *ct* and *hrt* values were much lower for the inferior oblique muscle than for the hindlimb muscles, it was found that the postnatal changes in *ct* and *hrt* were qualitatively similar in the inferior oblique and the fast gastrocnemius muscles, except at the ages above 10 weeks (Fig. 2 A, B). The *ct* and *hrt* at birth were approximately twice as large as the values obtained in the adult animal. In the inferior oblique the *ct* and the *hrt* continued to decrease beyond 20 weeks of age while the development with respect to speed of twitch contraction was completed within 6 weeks of age in the gastrocnemius muscles.

As seen in Fig. 2 A the reduction in *ct* of the inferior oblique muscle was more or less linear with age. The decline of the *hrt* (Fig. 2 B) was slower in the age period between 6 and 20

TABLE I Presentation of the groups of experimental animals in the study

Group	Age (days)	Weight (g)	Animals	Muscles
0 week	2 3	138–143	3	6
1 week	6–9	195–261	4	7
2 weeks	12 17	221 326	4	8
4 weeks	29–32	308–487	5	9
6 weeks	42–45	348–550	4	7
10 weeks	66–74	600–900	3	6
20 weeks	123 151	650, 1 200	2	4
Adult	over 365	800-3 500	3	5

medical recti and the retractor bulbi muscles were freed from the globe, which was enucleated The tituling membrane was raised to form a pool in the orbit, which was filled with warm mineral oil (37 C The body temperature wa monitored continuously and regulated to 37 38 C ith a heating pad the experiments parts of the muscles were removed for histochemistry

Isometric tension ecording The short tendon of the inferio oblique muscle was tied with braided to a hook glued to one end of a sensitive strain gauge (Endevco no. 8101). The other end of the strain was clamped t a metal base ttached to micromanipulator The undamped resonance frequency of the tension recording assembly was 2 kHz. The linear range of the system spanned from 10 mg to The signals from the strain gauge were amplified and displayed on a Medelec UV recording unit. The band of the mpl fiers was set at 0–10 kHz. In order to enable comparison bet een contractions ferent muscles, all tension recordings were made at the muscle length of maximal twitch response was taken t align the train gauge perpendicular to the natural pulling direction of the muscle.

St mulation. The muscle nerve was carefully dissected free from orbital tissue and cut as far as possible. It was positioned er paired pl tinum electrodes nd stimulated with square-wave pulses 0.1 ms duration, generated by Grass S8 stimulator and fed through an isolation un t.

Recording and measurement With some slight modifications the techniques have been similar to used by Hanson nd Lennerstrand (1977). *T tch responses* were recorded first t increasing intensity single pulse stimulation from threshold to supramaximal values. Threshold as about 0 4 V for the born a imals and decreased to about 0.2 V for dult cats. Stimulus intensities at least f e times the maximal twitch response were sed for the rest f the recordings of twitch and tetanic responses maximal amplitude of the twitch, the contraction time (t) and the half-relaxation time (hrt) of the contractio were measured. In addition the time for complete relaxation of the twitch as described in Results. *Tetanic responses* were elicited by trains of pulses ranging in frequency from 800 Hz. The *fusion frequency* f the tetanic stimulation was determined, i.e the lowest stimulus frequency to obtain a totally fused tension output with the present recording system. The minimum level of the sensitive tension device was just below 10 mg, which was sufficient for the determination of frequency even n the newborn muscles, with tetanus tension of 1 3 g. The *maximum tetanic tension* the *rate of tension rise* was measured for different stimulus frequencies. The rate of tension rise as the steepest slope of the ris g part of tetanic response. The values were expressed as the tension percent of the maximum tetanic tension per millisecond *Succinylcholine (Sch)* was injected i.v in of 1 mg per kg b.w and the contracture induced in the inferio blique by this drug was recorded animals were ventilated on pump d ring the transient paralysis of the respiratory muscles.

Statistical analysis. When both muscles i the same animal were used the values were lumped. An of variance (one way) was performed for each parameter studied n order t reveal significant differences between any of the age gro ps. For more detailed comparison between ge groups, confidence limits were calculated ccording to the method f multiple comparisons of Scheffé (1961). I ll graphic presentations of results mean values have been plotted. Th standard deviations for each parameter were of the same order of magnitude in all ge groups, nd could therefore be represented by the over all standard deviation from the analysis of variance This is given in the legend to each diagram.

Results

Twitch and tetanic tension Values for maximum twitch and tetanus tension produced by the inferior oblique muscle at various ages are given in Fig. 1 A and B, res ectively Tw tch



4. Changes in ct and hrt with increase in intensity of nerve stimulation. A shows the initial increase with stimulus intensity in muscle from new-born kitten and B the decrease in ct in the case of ...ek old animal. At supramaximal stimulus strengths ct levelled off. The parameters a, b and c from equation in the text are marked. These values and the regression lines calculated on computer ...g the method of least squares. In C mean slope values (b) for relations between stimulus intensity and ... hrt have been plotted for all age groups. The overall standard deviation ... ± 44.9 for ct and ± 103.8 ... hrt. The graph indicates that young muscles have positive slope and older muscles a negative slope. ... difference between age groups ... statistically significant only for ct.

...scles. The time for total twitch tension decline was estimated from double twitch responses ...stimulation at low repetition rates (10 Hz and below; see also Hanson and Lennerstrand ...7). At very low stimulus frequencies the first twitch had completely vanished before the ...ponse to the second stimulus appeared. The lowest frequency at which the two twitches ...rted to show summation has been plotted in Fig. 3 B for muscles of different ages. The ...ues for the newborn cat were the highest. Thus, in spite of the long hrt at this age, the ...line of the twitch response was faster than in older muscles. The difference between ...born and 6 weeks old animals was statistically significant ($p < 0.01$).

Twitch contraction and stimulus strength. On increasing the intensity of nerve stimulation ...m threshold to supramaximal values, more and more motor units were recruited in the ...scle, which lead to an increase in twitch amplitude and to changes in *ct* and *hrt*. The ...ter were studied in some detail, since it has been shown previously that *ct* and *hrt* of adult ...t eye muscle, in contrast to other skeletal muscle, had higher values at threshold than at ...ximal stimulus intensities (Lennerstrand and Bach-y-Rita 1974). This means that *ct* and ...t decreased with increasing stimulus strength in the lowest intensity range. Later as the ...ximal response level was approached, the twitch *ct* and *hrt* increased with stimulus in...nsity. The youngest muscles, on the other hand, showed a steady increase in both *ct* and

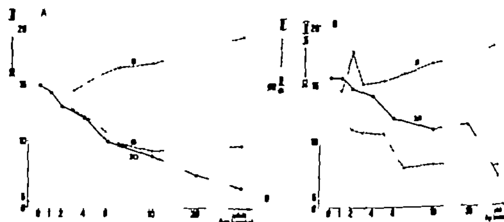


Fig. 2. Twitch contraction time (*ct*) in *A* and half-relaxation time (*hrt*) in *B* plotted against age in inferior oblique (IO), the gastrocnemius (G) and soleus (S) muscles. The values of G and S obtained the work by Hammarberg and Kellerth (1975) (courtesy of Dr J.-O. Kellerth). Note, that the left scale pertains to IO readings and the right scale to G and S values.

weeks than at earlier and later ages, but the difference in *ct* and *hrt* development was statistically significant. This was tested by calculating the *ct*/*hrt* ratio for each muscle comparing the values of the different age groups.

Twitch duration In Fig. 3 A, showing twitch responses from kitten eye muscles of age and 6 weeks and from an adult muscle, it can be seen that total twitch duration was shorter in the youngest than in the older muscles. Since the *hrt* was longer the younger the muscle (Fig. 2 B) it must be the latest phase of the twitch that tailed off more slowly in the older

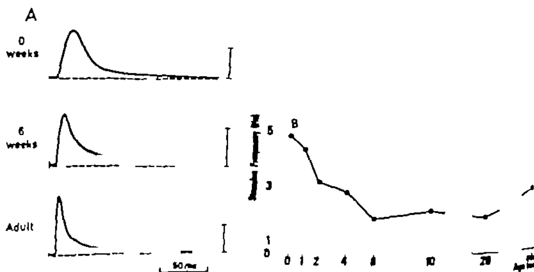
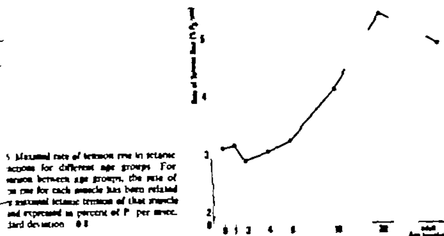


Fig. 3 A. Twitch responses obt ined in three different inferior oblique muscles of ages marked. Broken line indicates resting tension before stimulation. The decline of the twitch response is of longer duration the 6 weeks old animal and the adult cat than i the new-born animal. Bars to the right of traces indicate in top panel 0.2 g, and in middle and bottom panels 0.5 g. Same time scale in all recordings. B. Twitch duration estimated from the lowest stimulus frequency t produce summation between the twitches double stimulation. Note, that stimulus frequency was higher and twitch duratio thus shorter in young than in old kittens. Average values given for each age group. S.D ±0.9 Hz.



5 Maximal rate of tension rise in tetanic actions for different age groups. For parison between age groups, the rate of on rise for each muscle has been related e maximal tetanic tension of that muscle and expressed in percent of P per msec. dard deviation

bsolute alues for *ct* and *hrt* at threshold and at maximal activation are given in T ble II as found that the threshold *ct* but not *hrt* increased continuously with age

sion frequency As shown in Fig. 5 the fusion frequency was around 700 Hz in the born kitten and increased with age until the values saturated at about 325 Hz for muscles er than 6 weeks. The difference between values above 6 weeks of age and those below 2 weeks was statistically significant (p 0.01).

Maximal rate of tension rise Stimulus frequencies higher than the fusion frequency had be applied in all age groups in order to attain maximal rate of rise of the tetanic response. the newborn animal stimulation at 400 Hz was necessary and in the adult cat 600 Hz. The values of rate of tension rise in Fig. 6 have been expressed as the tension increase in cent of maximal tetanic tension (P_o) per millisecond. There was no significant variation ween muscles up to 6 weeks of age. At 10 weeks the alues started to increase and at 20 eks they were even higher than in the adult animal.

Effect of succinylcholine (Sch). This drug is known to cause a long-lasting contracture in traocular muscle of the adult cat, probably by inducing an extensive depolarization of the

BLE III Effects of succinylcholine on inferior oblique muscles at different ges. Drug action ha been expressed as succinylcholine-induced tension in percent of the maximal tetanic tension, and has been measured at the time of maximal succinylcholine effect and 5 and 10 min later. Mean alues (± S D) are given for each age group

Age	Ma effect	5 min	10 min
week	46 (9)	12 (±6)	1 7 (_1 6)
week	44 (±11)	6 (±6)	1 (±1)
weeks	38 (±7)	7 (±5)	3 (±3)
weeks	52 (±13)	15 (±11)	5 (+4)
weeks	48 (±11)	15 (±9)	4 (±5)
weeks	46 (±6)	5 (±5)	1 (±1)
weeks	41 (±3)	21 (±2)	14 (±2)
dult	44 (±6)	21 (±6)	13 (±6)

TABLE II Twitch *ct* and *hrt* obtained with threshold and supramaximal stimulation, respectively, at eye muscle of different age. Mean values (± S.D.) are given.

Age	*ct*		*hrt*	
	Threshold	Supramax.	Threshold	Supramax.
0 week	12.2 (±2.0)	15.3 (±2.9)	13.5 (±5.7)	16.0 (±3.0)
1 week	11.6 (±1.3)	14.4 (±0.8)	13.1 (±1.5)	18.8 (±4.8)
weeks	9.9 (±1.7)	13.1 (±0.8)	11.4 (±1.8)	14.8 (±2.7)
4 weeks	9.1 (±2.3)	12.0 (±0.9)	12.0 (±2.5)	13.0 (±1.5)
6 weeks	8.4 (±1.2)	10.0 (±0.8)	11.6 (±2.0)	12.0 (±1.8)
10 weeks	10.5 (±2.4)	9.0 (±1.5)	13.3 (±3.7)	11.0 (±1.5)
20 weeks	8.6 (±2.5)	7.0 (±0.21)	15.3 (±7.0)	10.8 (±2.1)
Adult	7.8 (±1.6)	6.0 (±0.8)	11.0 (±1.4)	7.4 (±1.6)

hrt with increasing stimulus strength as exemplified in Fig. 4 A for twitch *ct*. A change the adult pattern was observed in kittens 10–20 weeks of age (Fig. 4 B).

I order to compare twitch parameters in muscles of different age, the relation between the stimulus tensity and *ct* r *hrt* was approximated from the graphs as shown in Fig. 4 and expressed as follows

$$y = \begin{cases} a + bx & \text{if} \quad t \leq x \leq c \\ a & \text{if} \quad x > c \end{cases}$$

where *t* is threshold intensity intensity of reach maximal response level *a*, and *b* the slope of the (see Fig. 4 A). A positive slope denotes a increase in *ct* or *hrt* with stimulus strength and a negative the reverse An analysis of variance nd calculation of confidence intervals according to the method Scheffé (1961) was performed fo the *b*-values of muscles from cats of different age.

Significant differences were revealed for *ct* of kittens below ten weeks of age and cats. However there were no statistical difference between any age groups with respect *hrt* changes with stimulus intensity although the mean values of *ct* and *hrt* seem to vary similar way with age (Fig. 4 C). The standard deviation for *hrt* was much larger than for These findings strongly support the idea that the recruitment of motor units to increasing stimulus intensity followed different patterns in young and in adult eye muscles, with period of transition at 6–10 weeks.

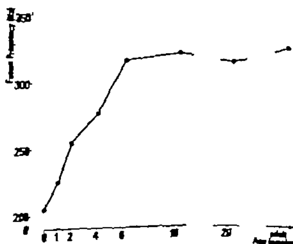
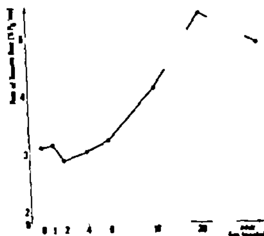


Fig. 5 Changes in fusion frequency of eye contraction w th ge. A erage lines plus standard deviation ±27.5 Hz.



Maximal rate of tension rise in tetanic ... tension for different age groups. For ... tension between age groups, the rate of rise for each muscle has been related ... maximal tetanic tension of that muscle ... and expressed as percent of P per msec. ... standard deviation 0.8 ...

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Discussion

Postnatal differentiation of fast and slow fibres

At birth the inferior oblique was a much slower muscle than in the adult cat. The speed o twitch contraction increased gradually over at least the first half year of the kitten's l In this respect the development of eye muscles was similar to that of fast limb although in the latter adult values were already obtained at the age of 2 months (Bu *et al* 1960 Buller and Lewis 1965 Mann and Salafsky 1970 Westerman *et al* 1973, F marberg and Kellerth 1975 a) However if speed of contraction in eye muscles instead estimated from the stimulus frequency to produce a fused response, the mature stage wa seem to be reached by 6 weeks. By using the rate of tension rise as the measure of contr tion speed, one would conclude that muscle development did not start until about 6 we of age and continued to approximately age 20 weeks.

The differences between the postnatal development of fusion frequency and twitch c traction time might be related to post-tetanic effects. The twitch responses reported in t paper were not influenced by previous muscle activity It will be shown in a subseq paper (Lennerstrand and Hanson 1978) that repetitive stimulation prolonged twitch rela tion and induced a staircase phenomenon in the tetanic response. These effects were m marked in the older animals, and could account for most of the levelling off of the cu relating fusion frequency to age.

In order to explain the developmental changes in the rate of tension rise of eye musc one might propose that the slow fibres matured more rapidly than fast fibres. Const values for the rate of tension rise during the first 6 weeks in spite of a decrease in tw contraction time would indicate that the slow fibre system completed most of its develo ment during this period The parallel changes in twitch contraction time and rate of tens rise up to age 20 weeks suggest that the fast fibres obtain their adult properties much la

Another finding to support the idea that the development of slow and fast fibres of e muscles followed different time courses was that the time for total twitch relaxation i creased from birth to six weeks of age, which would indicate that the slowest fibres, whi contribute most to the latest phases of the twitch response, not only remained slow fro birth but became still slower with age.

The eye muscle responses to threshold nerve stimulation

It was found that increasing the intensity of nerve stimulation from the threshold to supramaximal level affected twitch responses differently depending on the age of t In the young muscles, fast fibres seemed to be activated first an

..e and more slow fibres were recruited. The same pattern is seen in ordinary skeletal ..cle irrespective of age. However in cat eye muscle of age 10 weeks the response to ..shold stimulation as slower than those to supra threshold stimulus intensities. This ..ern is regularly seen in the adult muscle (Lennerstrand and Bach-y Rita 1974). The ..cle fibres responding at threshold must be innervated by large diameter nerve fibres, ..s these ould be activated by the lowest stimulus. It seems unlikely that the muscle ..es in question would be innervated by small nerve fibres in the young animal and later ..nervated by large nerve fibres, or that the nerve fibres to these muscle fibres should ..e grown at a faster rate than other nerve fibres. Fast hindlimb muscles are known to ..elop larger nerve fibres than slow muscle (Nyström 1968 b), as reflected also in the higher ..duction velocities of nerve axons innervating fast motor units than slow motor units ..gust *et al.* 1973, Bagust 1974). This relation holds also for kitten hindlimb muscle (Bagust, ..is and Westerman 1974). Since the number of muscle fibres seems to remain unchanged ..ing postnatal development (Westerman *et al* 1973), the most plausible explanation for ..e appearance in eye muscles of a slow response to threshold nerve stimulation at 10 weeks ..age would seem to be that some portion of the muscle fibres had changed their twitch ..perties and developed slightly slower contractions, in the same manner as the slow fibres ..the soleus muscle (*cf* Fig. A and B). These fibres may be located in the outer orbital ..yer since findings in the adult cat indicate that the motor response to threshold stimulation ..was in orbital fibres (Lennerstrand and Bach-y Rita 1974 Bach-y Rita 1975). The histo-..emical study of postnatal changes in eye muscles (Hanson *et al* 1977) has shown that the ..rbital layer is poorly defined at birth but fully developed at about 10 weeks of age

..ffects of succinylcholine

The succinylcholine experiments gave similar results at all stages of development. In response ..o an injection of the same amount of the substance per unit b.w., the muscles developed ..ntractures that amounted to 40–50 % of the maximal tetanic tension. The drug is thought ..o induce a long-lasting contraction of multiply innervated muscle fibres, *i.e.* the slow fibre ..components, by depolarizing the postsynaptic region of the muscle fibre membrane (Bach-y-..Rita 1971). In eye muscle of the adult cat the drug would seem to act on multiply innervated fibres of both the amphibian and the a..an types (Bach-y-Rita *et al.* 1977). The multiple ..innervation of adult extraocular muscle is probably only to a very small extent of the poly..neuronal type, with overlapping innervation from several nerve fibres to the same muscle ..fibre (Bach-y-Rita and Lennerstrand 1974). It is well-known, however that all fibres in ..kitten hindlimb muscle are polyneuronally innervated at birth, but that this type of innerva-..tion has disappeared at 6 weeks of age (Bagust, Lewis and Westerman 1973). The possibility of polyneuronal innervation of eye muscle fibres in young kittens makes it difficult to draw ..rm conclusions on the development of multiply innervated fibres on the basis of the suc..cinylcholine expts.

I wish to thank Stig Danielsson for advice and assistance in the statistical analyses. This study was supported by grants from Karolinska institutets fonder and the Swedish Medical Research Council (grants ..., 4774, 4719 and 3875).

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twitch muscle declined until adult patterns were reached at about 6 weeks (Hammarberg and Kellerth 1975 a). The motor units of slow twitch muscle remained moderately or highly fatigue resistant throughout development. In fast twitch muscle fatigue resistance decreased in units that developed into fast units but remained high in the units that became slow (Hammarberg and Kellerth 1975 c).

Post-tetanic potentiation (PTP) has not been systematically studied in adult eye muscles or during postnatal development. In hindlimb muscles PTP is known to be more pronounced in slow twitch than in fast twitch muscle at birth, the conditions reverse in adult animals, with clear PTP in the fast muscles and almost none in the slow ones (Standaert 1964, Nyström 1968). With regard to post-tetanic effects in individual motor units of adult hindlimb muscles, Olson and Swett (1971) reported that slow (S) units and fast, quickly fatiguing (FF) units show very little PTP but that PTP was prominent in fast, fatigue resistant (FR) units. PTP in motor units during postnatal development has not yet been thoroughly examined.

In view of the differencies between slow and fast fibres in other muscles, a study of fatigue and PTP might give further insight into the development of fast and slow components in eye muscles. A preliminary report on the results has been published elsewhere (Hanson, Lennerstrand and Nichols 1977).

Methods

General procedures used for activation of the inferior oblique muscle in kittens of known ages and the adult cat, and for recording of isometric tension responses have already been described (Lennerstrand and Hanson 1978). In this paper post-tetanic properties were studied in the following manner:

1. A conditioning stimulation of 200 Hz for 1 s was applied and the twitch response recorded at intervals during a post-tetanic period of about 10 min.
2. Continuous stimulations during 30 s at 50, 100, 200 or 300 Hz were applied in that order after which twitch responses were recorded for about 30 min. Measurements were made of tetanic tension, twitch tension, twitch contraction time (ct) and half-relaxation time (hrt) (Hanson and Lennerstrand 1977, Lennerstrand and Hanson 1978). EMG was recorded in a few instances using two thin platinum foil electrodes resting on each side of the muscle.
3. Test stimuli of 200 Hz for 0.5 s were used to examine the effects on the tetanic response of fatigue producing activation at a stimulus rate of 200 Hz for 30 s.

Results

PTP of the twitch response. Tetanic stimulation of a muscle over its nerve induces both fatigue and potentiation of the tension response. Potentiation can be of two types. Stimulations for several seconds at high rates are known to produce post-tetanic repetitive twitch activity in the nerve fibres, i.e. single pulse stimulation might lead to repetitive muscle contractions. This is often seen in slow but seldom in fast muscles of the cat hindlimb, at least in animals anaesthetized with barbiturates (Standaert 1964, Olson and Swett 1971). Shorter stimulations at lower rates give rise to post-tetanic potentiation (PTP) of the excitation-contraction mechanisms, seen most clearly in the twitch response of fast limb muscle (Brown and Euler 1938, Standaert 1964, Buller and Lewis 1965, Nyström 1968, Olson and Swett 1971, Hanson 1974).

In order to elicit PTP of the inferior oblique twitch response, a short (1 s) burst at 200 Hz

Acta physiol. scand. 1978. 103 144–153

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The postnatal development of the inferior oblique muscle of the cat

II Effects of repetitive stimulation on isometric tension responses

By

GUNNAR LENNERSTRAND and JERKER HANSON

Received 28 November 1977

Abstract

LENNERSTRAND G and HANSON, J *The postnatal development of the inferior oblique muscle of the cat II Effects of repetitive stimulation on isometric tension responses.* Acta physiol. scand. 1978 *103* 144–153

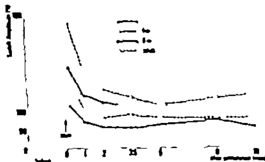
The changes with postnatal age in post-tetanic potentiation (PTP) and fatigue of the inferior oblique muscle have been studied in the cat. PTP of the twitch amplitude increased steadily with age up to 20 weeks after birth. Twitch contraction time (*ct*) and half relaxation time (*hrt*) was not significantly changed. The potentiation of the tetanic response also became more prominent with age The young muscles (10 weeks and below) were slightly more susceptible to fatigue than older muscles, but recovery was rapid in muscles of all ages. Intensive stimulation induced post-tetanic depression of twitch responses in muscles older than 1 week, but none in the muscles of new-born cats. *Ct* and *hrt* were greatly prolonged in muscles of 2–3 weeks of age. In these muscles, but never in the very youngest, repetitive firing could be observed in response to post-tetanic single nerve stimulation. The results are compared with those obtained in similar experiments on hind-limb muscles by other workers. They further support the idea raised in a previous paper that slowly muscle fibres develop quicker and reach maturity earlier than fast fibres

Key words: Postnatal development cat extraocular muscle fast and slow fibres, PTP fatigue

In a previous paper twitch and tetanic response properties of eye muscle were examined during postnatal development (Lennerstrand and Hanson 1978). Important differences were noted in the development of slow and fast fibre properties, and it was suggested that the slow fibres reached maturity earlier than the fast fibres. This paper concerns the variations with age in the effects of repetitive stimulation on isometric contraction, *i.e.* fatigue and post tetanic potentiation (PTP)

In the adult cat most motor units in eye muscles are fatigue resistant Only a few of the fastest units fatigue quickly (Lennerstrand 1974) However it is not known how fatigue properties change in eye muscles during development.

Both fast and slow hind limb muscles, which have been better studied in this respect than eye muscles, showed fairly high resistance to fatigue from birth to about three weeks of age. After that period the fatigue resistance of slow twitch muscle increased slightly while th



3. Post-tetanic effects on the twitch amplitude, expressed in percent of the pre-tetanic value, recorded at different times after a tetanizing stimulus of 200 Hz for 1 s. Average values are plotted for the age groups 0, 1 and 6 weeks and adult. Standard deviations: 0 w ±5.1, 1 w ±3.7, 6 w ±2.9, adult ±12.3

Fatigue and recovery of the tetanic response. Fatigue was induced by continuous stimulation for 30 s at 50, 100, 200 or 300 Hz. Figure 4 A displays the response to a 100 Hz stimulation of three different muscles: one from a newly born kitten and the others from a six week kitten and an adult cat.

In the older muscles the tension output increased steadily during the initial part of the stimulation, the so called stair-case phenomenon. The stair-case effect was seen at 50 and 100 Hz but not at 200 and 300 Hz. For the same rate of stimulation it was of longer duration in older animals than in young ones. This is seen in Fig. 5, which displays the relative tension in percent of the initial tension, recorded after 1, 3, 10 and 30 s of stimulation at various rates, in kittens of three different age groups and in the adult cats. An adult pattern in stair-case effects was attained at 20 weeks of age.

The tension remaining after 30 s of continuous stimulation, expressed in percent of the tension at stimulus initiation, was used as a measure of fatigue resistance. With stimulations at 50 Hz the stair-case effects dominated the response and no fatigue was recorded (Fig. 5). At 100 Hz the fatigue was much more pronounced in the young (0– weeks) than in the

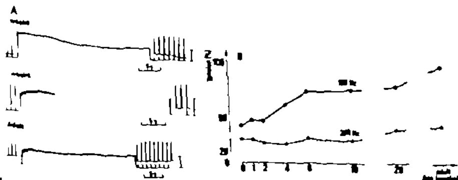


Fig. 4 A. The tetanic responses to 30 s, 100 Hz stimulation, preceded and followed by twitch responses, in muscles from a new-born (0 w), a six weeks old and an adult cat. Tetanic tension recorded on bottom trace, together with twitches at low gain. Top trace shows pre- and post-tetanic twitches at high gain. Tension bar (at extreme right) represents for 0 w: 5 g, 6 w: 10 g and adult: 20 g. B. Fatigue resistance to long stimulation for 30 s at 100 and 200 Hz in muscles of different ages. Fatigue resistance expressed as the tetanic tension remaining after 30 s continuous stimulation, in percent of the tetanic tension at stimulus initiation. Average values plotted; S.D. for 100 Hz stimulation: ±10.1% and 200 Hz ±9.4%.

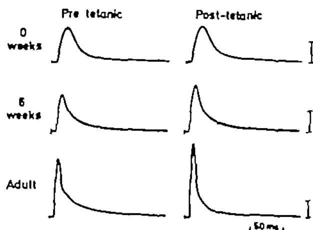


Fig. 1 Twitch contractions of muscles from a new-born (0 w), a 6 weeks old and a full grown cat, recorded pre-tetanically and 15 s after a potentiating tetanic 200 Hz stimulation of 1 sec duration. The tension scales (bars to the right) represent 0.2 g for the 0 and 6 w kittens, and 1 g for the adult cat.

was applied to the nerve. Fig. 1 shows the twitch response before and 15 s after the tetanization of a 2 day old, a 6 week old and an adult muscle. PTP occurred in all muscles but was much less pronounced in the newborn kitten. As seen in Fig. 2 A the immediate (15 s post stimulatory) effect on the twitch amplitude became increasingly larger with age. The difference between age groups below 4 weeks and above 20 weeks is significant at the 1 % level. The contraction time (*ct*) of the twitch response was only slightly affected. The half-relaxation time (*hrt*) was somewhat shorter than pre-tetanically at ages above 1 week (Fig. 2 B) but these variations were not statistically significant. The post tetanic changes in the twitch amplitude during a post-stimulatory period of 10 min are shown in Fig. 3. In the two youngest age groups the twitch amplitude was slightly reduced below the pre-tetanic value, after an initial potentiation lasting for about 1 min. The twitch did not fully recover in these muscles within the observation time (10–12 min). In the older animals (6 weeks and older) the twitch amplitude was potentiated for 2–5 min without post-stimulatory depression (Fig. 3).

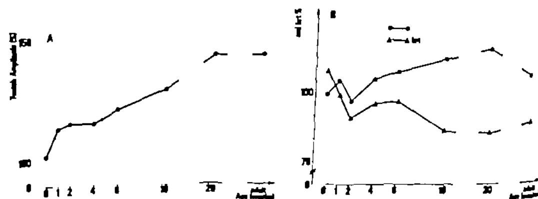
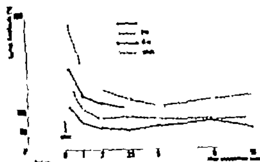


Fig. 2. Post-tetanic effects on the twitch response, induced by 200 Hz stimulation for 1 s, plotted against age. The amplitude (in A), *ct* and *hrt* (in B) have been measured in twitches recorded 15 s post-tetanically and have been expressed in percent of the corresponding parameter of the pre tetanic twitch. Average values for each age group are given. The standard deviation was about the same for all groups and the overall value calculated in an analysis of variance amounted to ±9.3% for twitch amplitude, ±8.1% for *ct* and ±8.3% for *hrt*.

3 Post-tetanic effects on the twitch ... expressed in percent of the pre... value, recorded at different times after ... stimulus of 200 Hz for 1 s. ... values are plotted for the age groups ... and 6 weeks and adult. Standard deviation 0 w: ± 5.1 ... 3.7 ... 6 w: ± 2.9 ... adult: ... 12 3



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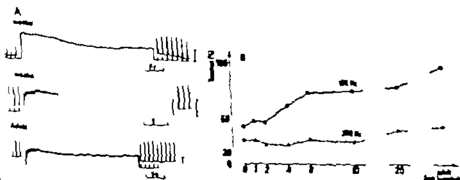


Fig. 4 A. The tetanic responses to 30 s, 100 Hz stimulation, preceded and followed by twitch responses, ... muscles from ... new-born (0 w), ... six weeks old and an adult cat. Tetanic tension recorded on bottom ... together ... with twitches at low gain. Top trace shows pre- and post-tetanic twitches at high gain. Tension bar (at extreme right) represents for 0 w: 5 g, 6 w: 10 g and adult: 20 g. B. Fatigue resistance to ... long stimulation for 30 ... at 100 and 200 Hz in muscles of different ages. Fatigue resistance expressed as the tetanic tension remaining after 30 ... continuous stimulation, in percent of the tetanic tension at stimulus initiation. Average values plotted; S.D. for 100 Hz stimulation, ± 10.1% and 200 Hz ± 5.4 ...

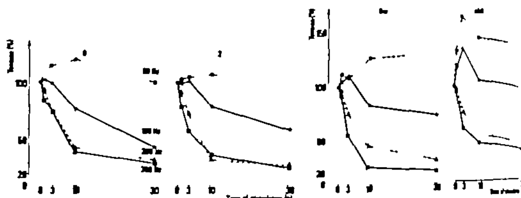


Fig. 5. Graphical display of tension development during continuous stimulations for 30 s at 50 (●), 100 (✳), 200 (▲) and 300 Hz (■). Tension measured after 1, 3, 10 and 30 s of stimulation and expressed in percent of tension at stimulus initiation. Average values plotted for the age groups of 0, 2 and 6 weeks and the adult animals.

adult cat (Fig. 4 B) but even here stair-case effects influenced the results. At rates of stimulation of 200 and 300 Hz, when no stair-case effect was seen, muscles in all animals below 10 weeks of age exhibited about the same amount of fatigue (Fig. 4 B). The 20 week old and adult muscles had a significantly higher fatigue resistance than the others ($0.01 < p < 0.05$).

The recovery of the tetanus response from fatigue was also followed. After a continuous 200 Hz stimulation for 30 s the response to a tetanic burst of 200 Hz for 1 s was lower than prior to the fatiguing stimulation. This is shown in Fig. 6 for a muscle of an adult cat. Within 1 min the magnitude of the tetanic response had returned to the value it had before the continuous stimulus was introduced. Figure 6 B displays graphically the recovery of the tetanic tension in muscles of different ages. Muscle tension was recorded at 2 s, 15 s and 1 min after the 30 s stimulation. The tension drop during the first seconds was most marked in the young animals but at all ages, except in the groups of 6 week and 10 week old animals, the recovery was complete in less than one minute. At 6 weeks the fatigue effect lasted for 5 min and at 10 weeks for 8–10 min. This difference was significant at the 5 % level.

Twitch responses after long stimulation. The effects of the long stimulations on the twitch response consisted of a mixture of potentiation and depression, probably of different time courses. This is exemplified in Fig. 7 A, where the magnitude of the twitch amplitude at different times after the conditioning stimulus (200 Hz for 30 s) has been plotted for muscles of new-born, 1 week old, 6 week old and adult cat. For all muscles except the very youngest (0 week), the twitch amplitude after an initial increase dropped markedly below the prestimulatory value. In the youngest muscles only potentiation and no depression of the twitch was seen (Fig. 7 A). It lasted for 5–8 min irrespective of the frequency of potentiating stimulus. In the muscles from animals older than 1 week, a depression of the twitch amplitude appeared within the first minute post tetanically and the tension returned slowly to the level before stimulation over the following 10–20 min. The time for full recovery of the twitch amplitude after a 200 Hz tetanization was about 12 min in 4 week old kittens and 15–20 min in 6, 10 and 20 week old kittens and the adult cat.

A post tetanic increase was seen in the *ct* and *hrt* values of all muscles as shown in Fig. 7 B and C for stimulations at 200 Hz for 30 s. *ct* and *hrt* returned to pre-stimulatory values

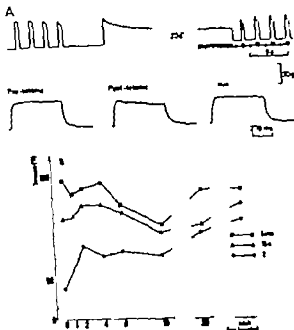


Fig. 6. Recovery from fatigue of the tetanic response to 200 Hz for 0.5 s. The test stimuli were tetanic trains at 200 Hz for 0.5 s and the fatigue stimulation 200 Hz for 30 s, exemplified for an adult preparation. A: traces show tetanic responses to test stimulations pre-tetanically and at 15 s and 1 min post-tetanically on an expanded time scale. In B is shown the recovery of the tetanic response in different age groups. The tetanic tension produced by the test stimulus as measured 2 s, 15 s and 1 min after the fatigue stimulation is expressed in percent of the pre-tetanic value. Recovery back to the 100%-level occurred rapidly in muscles of all ages, although the initial depression of the response was higher in young than in old muscle. Mean values given in B, standard deviation for curves at 2 s $\pm 8.8\%$, 15 s ± 5.2 and 1 min $\pm 5.7\%$.

within 10 min in the newly born and the one week old kitten, as well as in the adult animal. In animals 6 weeks of age the increase was larger than in the other age groups ($0.01 < p < 0.05$) and lasted longer (see Fig. 7 C). Similar patterns were observed for the 10 and 20 week old kitten.

In all age groups the largest twitch amplitude was obtained within 5 s from completion of continuous stimulation. The twitch response recorded at 15 s was analyzed with respect to age variations in amplitude, *ct* and *hrt* (Table I). The most prominent features were the large increases in *ct* and *hrt* in muscles above 6 weeks of age for conditioning stimulations at 200 and 300 Hz. These effects were less pronounced for 50 Hz stimulations but became clearly noticeable at 100 Hz as seen in Table I.

Double peaks were sometimes seen in the twitch curve during the first 2 min of the post tetanic period (Fig. 8 A). Such twitch responses were most common in the 6–10 week old muscles. They did occur also in the muscles of the adult cat, but they were never encountered in the newborn animals. As shown by the EMG-recording in Fig. 8 B the second peak must have been caused by repetive firing of the muscle in response to single shock stimulation of the nerve.

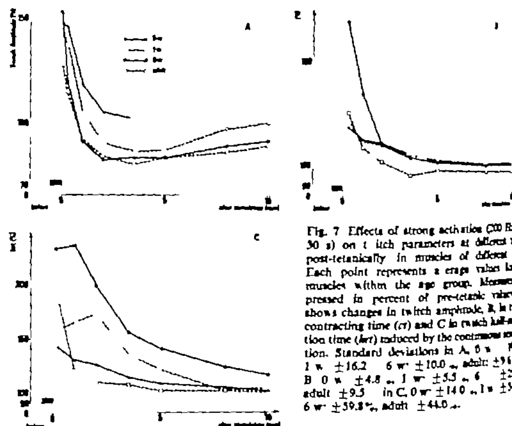


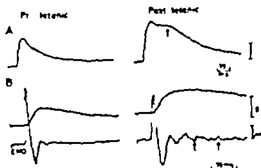
Fig. 7 Effects of strong activation (200 Hz, 30 s) on twitch parameters at different [illegible] post-tetanically in muscles of different [illegible] Each point represents average values for [illegible] muscles within the age group. Measures [illegible] pressed in percent of pre-tetanic values. A shows changes in twitch amplitude, B in [illegible] contracting time (*ct*) and C in twitch half-relaxation time (*hrt*) induced by the continuous stimulation. Standard deviations in A, 0 w [illegible] 1 w ±16.2 6 w ±10.0 adult ±9.1%, B 0 w ±4.8 1 w ±5.5 6 [illegible] adult ±9.5 in C, 0 w ±14.0 1 w ±34.7 6 w ±59.8%, adult ±44.0

Discussion

Fatigue properties In the adult cat eye muscles are known to be highly fatigue resist[illegible] (Lennerstrand 1974 1975) Eye muscles in the kitten showed a slightly lower endura[illegible] than adult muscle to the most intensive activations. The fatigue resistance remained at [illegible]

TABLE I. Twitch parameters measured 15 s after repetetive stimulation for 30 s at different rates, in perc[illegible] (± S.D.) of pretetanic values.

Age (weeks)		0	1	2	4	6	10	20	Adult
50 Hz	n	6	6	6	8	7	7	4	3
	ampl	149 (±35)	119 (±[illegible]5)	127 (±19)	124 (±8)	113 (±13)	134 (±10)	159 (±6)	141 (±[illegible]
	ct	1[illegible]3 (±11)	118 (±8)	116 (±8)	126 (±7)	124 (±13)	129 (±8)	133 (±13)	114 (±[illegible]
	hrt	133 (±15)	137 (±21)	133 (±23)	131 (±13)	146 (±41)	107 (±14)	103 (±[illegible]6)	112 (±[illegible]
100 Hz	n	6	6	6	9	7	6	4	4
	ampl	131 (±16)	121 (±22)	127 (± [illegible]1)	117 (±19)	112 (±11)	126 (±19)	134 (±6)	121 (±[illegible]
	ct	117 (±6)	121 (±13)	134 (±17)	146 (±30)	167 (±45)	149 (±14)	135 (±14)	139 (±[illegible]
	hrt	137 (±[illegible]5)	172 (±69)	151 (±35)	154 (±31)	194 (±29)	1[illegible]8 (±30)	168 (±47)	1[illegible]4 ([illegible]
200 Hz	n	6	7	8	9	7	6	4	3
	ampl	146 (±19)	138 (±[illegible]3)	1[illegible]5 (±22)	130 (±31)	130 (±16)	113 (±[illegible]3)	144 (±13)	120 (±[illegible]
	ct	118 (±5)	121 (±7)	121 (±10)	145 (±35)	169 (±22)	153 (±19)	135 (±13)	123 ([illegible]
	hrt	141 (±33)	158 (±66)	143 (±23)	154 (±[illegible]9)	233 (±68)	[illegible]22 (±58)	184 (±63)	184 (±[illegible]
300 Hz	n	4	5	6	8	7	7	4	3
	ampl	153 (±21)	150 (±19)	130 (±[illegible]2)	140 (±21)	140 (±14)	135 (±11)	157 (±7)	1[illegible]6 (±[illegible]
	ct	117 (±5)	120 (±8)	121 (±13)	144 (±23)	168 (±21)	144 (±10)	149 (±9)	114 ([illegible]
	hrt	142 (±13)	153 (±67)	141 (±37)	164 (±19)	[illegible]23 (±37)	216 (±75)	[illegible]07 (±55)	183 (±[illegible]



[Fig.] 3 A. Post-tetanic twitch response in [.. w]eeks old cat recorded 15 [s] after potentiat[ing .. t]rain 30 [s] 200 Hz stimulation showing [doub]le peaks (arrow) absent in the pre-[teta]nic twitch response. B. T[w]itch response [and] EMG recorded pre- and post-tetanically [i]n adult cat. Repetitive firing is seen post-[tetan]ically (arrows) after a 30 [s] stimulation [at 2]00 Hz

[..]e level from birth up to 20 weeks of age when adult values were reached. Strong muscle [co]ntractions lasting for minutes might reduce the blood flow to the muscle and render it [is]chemic, thereby influencing the normal fatigue properties (Hammarberg and Kellerth [19]75 a). It cannot be excluded that such effects might have been introduced in the youngest [mu]scles by the 30 s stimulations, but in the older muscles and in the adult cat the rates and periods of constant muscle fibre activation do probably not exceed those that occur [un]der natural conditions (cf. Yamanaka and Bach-y-Rita 1968, Lennerstrand 1974). The [rec]overy from fatigue after the long-lasting stimulation was rapid at all ages, except at 6 [we]eks and 10 weeks of age.

With the lower stimulus rates used (50 and 100 Hz) potentiation of the tetanic response [oc]curred. This gave rise to a stair-case phenomenon that reduced or even concealed the [fa]tigue effects of the long-lasting stimulation. The stair-case effect was more prominent in [th]e 6 week or older animals than in the younger kitten.

Adult cat eye muscle has a mixed population of slow and fast motor units. All of the slow [u]nits and the majority of the fast units were highly fatigue resistant (Lennerstrand 1974). If [th]e total tension response of eye muscles at all ages reflected the combined activity of in-[di]vidual motor units as directly as in hindlimb muscle (Hammarberg and Kellerth 1975 b, [c]), the proportion of fatigue resistant to fatigue sensitive units would seem to rise slightly [w]ith age, at least during the first 20 weeks of postnatal development.

PTP and development of fast and slow fibres. In a previous paper (Lennerstrand and [H]anson 1977) evidence was presented in support of the idea that slow fibres in cat eye muscles [w]ere more or less fully developed at 6–10 weeks of age, while the fast fibres did probably not reach maturity until the cat was 1/2–1 year old. PTP of the twitch response might provide another means by which differences in the rate of maturation between fast and slow fibres can be studied.

Eye muscles contain slow fibres that are multiply innervated and show several end-plate regions on the same fibre (Bach-y-Rita 1971, 1975, Lennerstrand 1974, Alvarado and van Horn 1975), while all slow fibres of hindlimb muscles are focally innervated with only one end-plate region per fibre. Unfortunately there are no studies available on PTP in slow multiply innervated fibre systems, neither in the mature nor in developing muscles. Provided [th]e PTP effects in the multiply innervated fibres of eye muscles are similar to those of slow [fi]bres in hindlimb muscles, the PTP results in developing eye muscles could be interpreted as

follows (i) PTP increased in eye muscles up to 20 weeks of age, indicating that fast fibre properties were developing over this period (Buller and Lewis 1965 Nyström 1968). The change occurred in parallel with the increase in speed of twitch contraction, used in a previous paper (Lennerstrand and Hanson 1978) as a sign of fast fibre development, (ii) when strong and long lasting activations were applied the shape of the twitch response changed due to post-tetanic repetitive firing in the muscle. These effects were not seen in the newborn animal but became more and more marked in older muscles and reached their maximum at 6–10 weeks of age Repetitive firing has been observed only in slow muscle (Standaert 1964, Nyström 1968 Olson and Swett 1971 Stephens and Stuart 1975). The findings in eye muscles would indicate that slow fibres were more or less fully developed at age 10 weeks, and that later changes in muscle characteristics would be caused by the further development of fast fibres

The early development of the slow fibre system in eye muscles may be connected with the demands for slow movements and sustained motor activity in fixation and binocular vision. The visual functions of the cat reach maturity around the age of 10 weeks (Blakemore and van Sluyters 1975) and at this age the slow fibre component of extraocular muscle would seem fully developed

We wish to thank Stig Danielsson for advice and assistance in the statistical analysis. This investigation was supported by grants from Karolinska Institutets fonder and from the Swedish Medical Research Council (grants No. 4751 4719 and 3875).

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Binding of thiamine to nicotinic acetylcholine receptor in *Torpedo marmorata* and the frog end plate

By

LENNART WALDENLIND LENA ELFMAN and BO RYDQVIST

Received 29 November 197

Abstract

WALDENLIND L. L. ELFMAN and B RYDQVIST *Binding of thiamine to nicotinic acetylcholine receptor in Torpedo marmorata and the frog end plate* Acta physiol. scand. 1978. 103 154–159

Thiamine was found to bind to the isolated nicotinic receptor (nAChR) from *Torpedo marmorata* ($K_D = 3–5 \times 10^{-8}$ M). The binding was reversible and inhibited by α-neurotoxin from *Naja naja siamensis*. Acetylcholine binding was not inhibited by thiamine. When thiamine (5 10^{-4} M) was applied to sartorius muscles a decrease of miniature end plate potential amplitudes was seen. This effect was reversible after the perfusion fluid of the muscle was changed to normal Ringer solution. Thus biological and electrophysiological studies indicated a binding of thiamine to the nAChR.

Abbreviations ACh = acetylcholine, MEPP = miniature end plate potential, nAChR = nicotinic acetylcholine receptor and PTP = post-tetanic potentiation

It has been shown that post tetanic potentiation (PTP) is abolished in thiamine deficiency induced by pyrithiamine (Waldenlind 1977). The PTP was then recovered by thiamine injections. The importance of thiamine for PTP was not correlated to the cocarboxylase functions of thiamine-diphosphate. Besides it was shown that d tubocurarine in doses (4 µg) which left the ordinary supramaximal muscle twitches unaffected, blocked the PTP. Therefore it was assumed that thiamine was necessary for curare sensitive receptors mediating PTP. This assumption led us to study the binding of thiamine to the purified nAChR from *Torpedo marmorata* and the electrophysiological effects of thiamine on miniature end plate potentials (MEPP's) registered from the muscle of the frog *Rana pipiens*

Methods

Binding of thiamine to nAChR

The nAChR from *Torpedo marmorata* electric organ was purified by biospecific chromatography on acetylated *Naja naja siamensis* toxin bound to Sepharose 4 B (Heilbronn and Mattsson 1974). Carbachol elution gave a protein peak, which was further purified on a DEAE-ion exchanger in order to get rid of carbachol and traces of acetylcholine esterase.

[illegible] of (^{35}S)thiamine hydrochloride (10^{-4}–10^{-8} M) to the nAChR [illegible] tested [illegible] earlier described, both the Sartorius membrane filter method (Heilbronn and Mattson 1974) and equilibrium dialysis [illegible] et al. 1974). The nAChR (1 mg/ml) [illegible] incubated [illegible] with neurotoxin α-neurotoxin, specific [illegible] for nAChR, for 15 min at room temperature before the binding of thiamine [illegible] studied. [illegible] one experiment the receptor [illegible] incubated [illegible] with oxythiamine and pyrithiamine (up to 10^{-3} M) prior to [illegible]-binding studies. Oxythiamine inhibits cocarboxylase function of thiamine-diphosphate but never [illegible] neurological symptoms [illegible] pyrithiamine induces neurological symptoms [illegible] of thiamine [illegible]ency [illegible] without any major direct effect on cocarboxylase functions of thiamine-diphosphate (Sheyn[illegible] 1967)

[illegible] study [illegible] whether thiamine could inhibit the binding of ACh, the receptor protein [illegible] first incubated [illegible] with [illegible] M Sarin for 60 min [illegible] room temperature in order to block traces of acetylcholine esterase. Thiamine [illegible] M) [illegible] then added and the incubation continued for another 30 min. (^{3}H)ACh (10^{-6} M) in [illegible] containing buffer [illegible] then added and equilibrium dialysis [illegible] performed. [illegible] was determined according to Lowry *et al.* (1951).

[illegible] recordings

[illegible] recording of MEPP [illegible] performed on sartorius muscle fibres from small *Rana pipiens*. The muscle [illegible] dissected free and placed in [illegible] perspex chamber (volume 5 ml) [illegible] with its deep surface upward. The Ringer [illegible] contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2.15 mM Na_2HPO and 0.85 mM NaH_2PO_4, the pH [illegible] 7.0–7.2 (Adrian 1956). The solution in the chamber [illegible] changed carefully [illegible] with continuous [illegible] from muscle fibres. The location of the end-plate region [illegible] determined by [illegible] visual inspection [illegible] binocular zoom microscope [illegible] 100 [illegible] with dark field illumination. Intracellular potential changes were [illegible] using glass micropipettes filled [illegible] with 3 M KCl and [illegible] with [illegible] tip resistance of 8 10 MΩ. The micro[illegible] was connected to [illegible] high input impedance ([illegible] 10) amplifier. The output from this amplifier [illegible] fed [illegible] an oscilloscope (Tektronix 5103 N), [illegible] digital [illegible] (to record resting membrane potential) and [illegible] 250 amplifier (AC-coupled, [illegible] with lower cut-off frequency ([illegible] 3 dB point) of 0.3 Hz) the output [illegible] which was fed to [illegible] tape-recorder (Hewlett-Packard 3960). The speed of the tape-recorder [illegible] set to [illegible] inch [illegible] which [illegible] equivalent to [illegible] bandwidth of 0–1250 Hz. The MEPP [illegible] thus stored on tape could later [illegible] displayed on an oscilloscope and recorded on photographic film.

Materials

[illegible]ACh (250 mCi/mmol) and (^{35}S)thiamine (150 mCi/mmol) [illegible] were purchased from the Radiochemical [illegible], Amersham, U.K. Oxythiamine and pyrithiamine were obtained from Sigma Chemical Co. Other [illegible] were from usual commercial sources.

Results

[illegible] binding of thiamine to nAChR

[illegible] purified nAChR protein from *Torpedo marmorata* which has a specific affinity for [illegible] cholinergic ligands (Chang and Lee 1966, Heilbronn and Mattson 1974, Meunier *et al.* [illegible] 1974) is also able to bind thiamine *in vitro* (Fig. 1 and 2). The K_D-value for the binding of [illegible] thiamine was shown to be 3.05 ± 0.87 [illegible] 10^{-6} M when measured by the Sartorius membrane [illegible] filter technique (Fig. 1) and 5.10 ± 2.63 [illegible] 10^{-6} M when measured by equilibrium dialysis [illegible] (Fig. 2). The maximum binding capacity was 0.84 ± 0.10 nmol/mg receptor protein when [illegible] the Sartorius filter technique was used (Fig. 1) and 2.57 ± 0.79 nmol/mg protein when [illegible] measured by equilibrium dialysis (Fig. 2). All values are mean ± S.D.

The binding of thiamine to the nAChR was inhibited by the nAChR antagonist [illegible]-neuro[illegible]toxin from *Naja naja siamensis* at a concentration of 10^{-7} M. This was proven by both [illegible] techniques. The binding of ACh (10^{-6} M) was not inhibited by thiamine (10^{-4} [illegible] 10^{-8} M), nor [illegible] did the presence of thiamine (10^{-4} M) change the K_D-value (2.3 [illegible] 10^{-8} M) for the binding of ACh (checked by equilibrium dialysis).

Acta physiol scand. 1978. 103. 154–159

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Binding of thiamine to nicotinic acetylcholine receptor in *Torpedo marmorata* and the frog end plate

By

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Received 29 November 1977

Abstract

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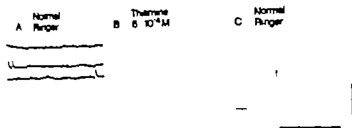
Abbreviations. ACh = acetylcholine MEPP = miniature end plate potential, nAChR = nicotinic acetylcholine receptor and PTP = post-tetanic potentiation.

It has been shown that post tetanic potentiation (PTP) is abolished in thiamine deficiency induced by pyrithiamine (Waldenlind 1977). The PTP was then recovered by thiamine injections. The importance of thiamine for PTP was not correlated to the cocarboxylase functions of thiamine-diphosphate Besides it was shown that d-tubocurarine in doses (4 µg) which left the ordinary supramaximal muscle twitches unaffected blocked the PTP Therefore it was assumed that thiamine was necessary for curare-sensitive receptors mediating PTP This assumption led us to study the binding of thiamine to the purified nAChR of *Torpedo marmorata* and the electrophysiological effects of thiamine on miniature end plate potentials (MEPP's) registered from the muscle of the frog *Rana pipiens*

Methods

Binding of thiamine to nAChR

The nAChR from *Torpedo marmorata* electric organ was purified by biospecific chromatography on acetylated *Naja naja siamensis* toxin bound to Sepharose 4 B (Heilbron and Mattson 1974). Carbachol elution gave a protein peak, which was further purified on DEAE-ion exchanger in order to get rid of the carbachol and traces of acetylcholine esterase.



3. Effect of thiamine on MEPP's. A. Before application of thiamine. B. Two min after application of ...ine (5 × 10⁻⁴ M). C. After return to Normal Ringer. Resting membrane potential: (A) −93 mV (B) ... mV (C) −94 mV. Time bar: 0.5 s; Vertical bar: 2 mV

...he resting membrane potential was not affected in thiamine solutions, the values ranging ...n about −90 mV to −95 mV. After the final measurements in normal saline, the micro...ode was withdrawn from the fibre and the resting membrane potential was found to be ...4 mV

Discussion

Isolated nAChR from *Torpedo marmorata* binds thiamine at a concentration of 10^{-4} M ... binding of thiamine is prevented by α-neurotoxin from *Naja naja siamensis*, which is a

A Normal Ringer — B Thiamine 5 10⁻⁴ M — C Normal Ringer — D Thiamine 5 10⁻³ M — E Normal Ringer (mV)

Fig. 4. Histograms from experiments as illustrated in Fig. 3 showing distribution of amplitudes of MEPP's in ... muscle fibre treated with two concentrations of Thiamine (5 × 10⁻⁴ M and 5 × 10⁻³ M). n number of ...fibres, f mean MEPP frequency (s⁻¹), m ± mean ± standard deviation of MEPP amplitude (mV). Resting membrane potential. (A) −92 mV (B) −90 mV (C) −93 mV (D) −95 mV (E) −94 mV

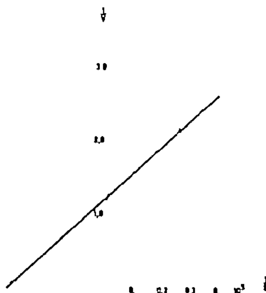


Fig. 1

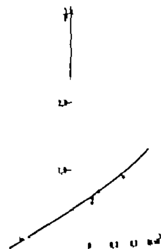


Fig. 2

Fig. 1 Lineweaver Burk plot showing binding of thiamine to nAChR using the Sartorius membrane method. The K_D-value calculated from the curve is $3.05 \pm 0.87 \times 10^{-4}$ M and the maximal binding cap. 0.84 ± 0.10 nmol of thiamine per mg of protein.

Fig. 2. Lineweaver Burk plot showing binding of thiamine to nAChR using equilibrium dialysis. The K_D value calculated from the curve is $5.10 \pm 2.63 \times 10^{-4}$ M and the maximal binding capacity 2.57±0.. of thiamine per mg protein.

Oxythiamine and pyrithiamine (two thiamine antagonists) were tested in order to determine whether thiamine binding to nAChR could be prevented. No inhibition could be seen at concentrations up to 10 M

Electrophysiological recordings

Thiamine (10^{-4}–5×10^{-4} M) was applied to frog sartorius muscles for different periods of time (2–10 min mainly depending on the concentration used). Up to a concentration of 10^{-4} M no changes in mean amplitude of the MEPP's could be seen. At higher concentration (1–5×10^{-4} M) a decrease in MEPP amplitude was observed (Fig. 3). Amplitude histograms for spontaneous MEPP's in the same fibre as in Fig. 3 are shown in Fig. 4 at two concentrations of thiamine (5×10^{-4} and 5×10^{-4} M). At 5×10^{-4} M the mean amplitude was unchanged (at 95% significance level) compared with the mean amplitude in control measurements both before and after application of thiamine (Fig. 4 A B and C). After application of 5×10^{-4} M thiamine, the mean MEPP amplitude was drastically reduced from 0.9 mV (Fig. 4 C) to 0.3 mV (Fig. 4 D). At this concentration, reduction occurred within about 1 min, the total time of application being 3 min. After 5 min in normal Ringer solution the MEPP amplitude had returned to the same value (0.9 mV) (Fig. 4 E) as before application of thiamine (no difference at a 95% significant level). The MEPP frequency seems to be somewhat increased at both concentrations of thiamine compared with the frequency in Ringer solution (Fig 4).

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specific antagonist of the nAChR (Chang and Lee 1966). The binding of thiamine to isolated nAChR occurs in a similar dose range to that which inhibits nicotine-induced contractions of the guinea-pig ileum (Waldenlind 1977) The K_D-value for thiamine in the present study is based on *in vitro* experiments. The binding constants need not be the same *in vivo* It is known that the binding of *e.g.* ACh to the isolated nAChR occurs at a level which is higher than that which is active on isolated organ preparations. Extrinsic factors such as temperature, pH intact membrane structure and possible co-factors present, etc. are defined in the non-injured tissue, may change the binding constant. Therefore the K_D value gives no information concerning the concentration of thiamine which could be active *in vivo* Thiamine decreased the mean MEPP amplitude in the frog end plates without affecting the membrane potential when added in a concentration of $5 \cdot 10^{-4}$ M (Fig. 3). At this concentration about 85% of all thiamine binding sites of the nAChR from *Torpedo marmorata* are occupied This electrophysiological effect may therefore be explained as being due to binding of thiamine to the nAChR of the end plates. The bulk concentration of thiamine ($5 \cdot 10^{-4}$ M) necessary to block the MEPP's is probably higher than the concentration at the end plate region due to the high polarity of the thiamine molecule which makes it difficult to penetrate tissue barriers.

In the purified nAChR the high affinity site ($K_D = 3 \cdot 10^{-8}$ M) is changed to a low-affinity site ($K_D = 2 \cdot 10^{-6}$ M) for ACh Thiamine could not inhibit the low-affinity binding of ACh to purified nAChR but in electrophysiological studies where only the high-affinity site is present, the results indicate an effect on either the acetylcholine binding or the ionophore activity of the nAChR.

The electrophysiological studies do not reveal any certain physiological role for thiamine at the post-synaptic nAChR. However it seems clear that thiamine binds to nAChR as this has been shown by binding studies on isolated receptors and by electrophysiological recordings. PTP is mediated by the activation of presynaptic curare-sensitive receptors and thiamine is necessary for the function of these receptors (Waldenlind 1977 Waldenlind, To be published) It is thus possible that the physiological role of thiamine may be in the mediation of PTP through activation of curare-sensitive receptors.

We wish to thank Professor E. Heilbronn for helpful comments on the manuscript.

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1 Effect of hexamethonium on PTP. The first arrow indicates the infusion of hexamethonium (1.0 mg/kg). Part of the PTP is still left. No effect on the single twitches is seen. The next PTP is, however, significantly smaller. The second bar indicates the infusion of an additional dose of hexamethonium (0.5 mg/kg). PTP is now totally abolished and the single twitches are still the same as in the beginning. The resistance [illegible] traction force [illegible] equivalent to 55 g.

Methods

In Sprague-Dawley rats (250–300 g) were anaesthetized with mebumal sodium (30 mg/kg). The trachea was intubated with a plastic tube just below the larynx. The external jugular vein was cannulated to allow injections. The normal blood circulation of the masseter muscle was intact. The masseteric nerve was prepared and cut off proximally. Supramaximal stimulation (1.5 times that required for maximal stimulation) of the masseteric nerve was then carried out both directly and by field stimulation (0.2 Hz, 0.2 ms) by Grass SD 9 stimulator using platinum electrodes. The isometric twitches of the masseter muscle were measured by a thread through the mandibular bone. The thread was connected to a Grass force-displacement transducer (FT 10) and a Grass polygraph (model 7 B). The contraction force registered is therefore proportional to the tension in the masseter muscle. The initial tension was equivalent to 150 g. The contraction [illegible] given in the individual figures. The PTP was provoked by a brief tetanic stimulation at [illegible] Hz for 5 s. [illegible] the contracted muscle responded with individual twitches at frequencies at 20 Hz (typical) for [illegible] [illegible] the term PTP is somewhat incorrect. However it is an accepted way of describing the phenomenon [illegible]. All substances were dissolved in 150 mM NaCl (0.1 ml) and injected.

Materials

[illegible] chloride and hexamethonium bromide were obtained from Sigma Chemical Co [illegible] and sodium pentobarbital from ACO, Sweden, prostigmin and diazepam from Hoffman LaRoche, [illegible] [illegible] from Vitrum, Sweden and haloperidol from LEO, Sweden.

Results

Hexamethonium inhibited PTP dose-dependently. The ordinary twitches were unaffected (Fig. 1). Prostigmin (1.5 µg/kg) did not reverse the effect of hexamethonium, on the contrary a potentiation of the hexamethonium effect was noted. PTP was inhibited by doses of hexamethonium (ED_{50} 1.75–2.0 mg/kg, n = 4) slightly higher than those inhibiting the action of nicotine on the isolated guinea-pig ileum (Waldenlind 1977).

Succinylcholine blocked PTP dose-dependently in doses (ED_{50} ~3–7 µg/kg, n = 4, ED_{100} ~ 9–13 µg/kg, n = 4) which did not affect the ordinary muscle twitches (Fig. 2). At higher doses the ordinary muscle twitches were reduced (ED_{50} ~ 40–50 µg/kg, n = 4). The effect of succinylcholine on the PTP could not be reversed by prostigmin. The ordinary muscle twitches increased after prostigmin administration at a dose when the PTP was totally inhibited by succinylcholine showing that the end plate receptors were unaffected (Fig. 2).

[illegible]

Acta physiol. scand. 1978. 103. 160–164

From the Department of Pharmacology Karolinska Institutet, Stockholm, Sweden

Pharmacological properties of curare-sensitive receptors mediating post tetanic potentiation in rat masseter muscle

By

LENNART WALDENLIND

Received 29 November 1977

Abstract

WALDENLIND L. *Pharmacological properties of curare-sensitive receptors mediating post tetanic potentiation in rat masseter muscle* Acta physiol. scand. 1978. *103* 160–164

Post-tetanic potentiation (PTP) of single muscle twitches after "tetanic" stimulation at 1 to 20 Hz in masseter muscle has been studied. PTP was inhibited by d-tubocurarine (?–6 μg/kg), hexamethonium (1.75–2.25 mg/kg), succinylcholine (5–7 μg/kg), prostigmin (1.0–1.5 μg/kg), lidocain (3–4 mg/kg) and ... (215–240 μg/kg). The doses did not affect the control twitches. Prostigmin could not reverse the blocking effect on the PTP caused by these drugs. Atropin was without any effect on the PTP. The nicotinic receptors mediating PTP differ from both end-plate and ganglionic receptors since drugs blocking both types of receptors are effective. The sensitivity to the blocking agents is higher or the same (in the case of hexamethonium) as for the two other types of nicotinic receptors. Thus the PTP is dependent on activation of a "nicotinic" receptor active during physiological conditions, suggesting the presence of an additional mechanism for neuromuscular transmission in rat twitch fibres.

Tetanic stimulation of fast skeletal muscle causes a transitory increase in the peak tension of the isometric twitch response (post tetanic potentiation, PTP), whereas a decrease is noted in slow mammalian muscles (Brown and Euler 1938, Bowman 1962, Standaert 19.. and Close and Hoh 1968). PTP is absent in muscles perfused with saline solutions (Brown and Euler 1938) suggesting the importance of a humoral factor. It has been shown that PTP is abolished in thiamine-deficiency induced by pyrithiamine, or by d-tubocurarine in doses lower than those affecting the normal single twitches (Waldenlind In Press). Since thiamine has been shown to bind to nicotinic receptors (Waldenlind *et al* In Press) and since the role of thiamine for the development of PTP was found to be unrelated to the co-carboxylase functions of thiamine-diphosphate, it was thought that thiamine is important for the function of curare-sensitive receptors necessary for the occurrence of PTP. In the present article the pharmacological properties of curare-sensitive receptors mediating PTP are studied.



·3 The effect of atropin on *Atropin (75 mg/kg) has been ·d after the first tetanus and ffect on either the single ·es or the PTP can be seen. ·etanus contraction force is ·alent to 63 g.

Discussion

results show that PTP is inhibited by nicotinic receptor blocking agents in doses lower those affecting the ordinary muscle twitches. Inhibition of PTP occurred both with ·ubocurarine and hexamethonium indicating that the "nicotinic" receptors mediating are undifferentiated and thus constitute a third class of nicotinic receptors active ·ing physiological conditions. In Table I it is clearly seen that nicotinic receptors mediating P in fast mammalian twitch muscles are pharmacologically different from end plate ·eptors and nicotinic receptors mediating post-tetanic repetition in slow muscles, such as cat soleus muscle. This difference is also seen in Fig. 2 where PTP is totally inhibited, t still prostigmin could increase the ordinary twitches. The end plate receptors must ·refore be unaffected since succinylcholine blocks ordinary muscle twitches irreversibly the case of ouabain and lidocain, the single twitches could always be increased even if ·P was fully inhibited. Since the increase in single twitches after prostigmin treatment is ·e to post-tetanic repetition, PTP and post-tetanic repetition seem to have different pharma·logical characteristics.

It is probable that the action of lidocain is on nicotinic receptors since a small dose of ·tubocurarine which has no effect itself on the PTP potentiates the action of lidocain or ·abain. Presynaptic nicotinic receptors have been isolated which bind both local anesthetics ·nd ouabain in low concentrations (Denburg and O'Brien 1973).

·ABLE I The table summarizes the effects of drugs on end plate receptors, nicotinic receptors mediating PTP in fast mammalian muscles and nicotinic receptors mediating post-tetanic repetition. The effects of drugs on post-tetanic repetition are from Raker (1969).

	end plate receptors	PTP	post-tetanic repetition
d-tubocurarine	reversible inhibition	nonreversible inhibition	reversible inhibition
hexamethonium	0	nonreversible inhibition	0
succinylcholine	depolarizing nonreversible	nonreversible inhibition	stimulation
prostigmin	stimulation (by AChE-effect)	nonreversible inhibition	stimulation
atropin	0	0	0
lidocain	inhibition	inhibition	inhibition
nembutal	0	0	depression
ouabain	0	nonreversible inhibition	?



Fig. 2. Effect of succinylcholine and prostigmin on the PTP. The first arrow indicates the administration of succinylchol ne (10 μg/kg). The PTP is almost completely abolished. The second bar shows the infusion of prostigmin (6 μg/kg). The single twitches at low frequency stimulation increases but PTP is still abolished. The pretetanic contraction force is equivalent to 44 g.

Lidocain also inhibited the PTP dose-dependently (ED_{50} = 3–4 mg/kg, n = 5) as did ouabain (ED_{50} = 215–240 μg/kg, n = 5) (Fig. 3). Both lidocain and ouabain completely inhibited the PTP without any effect on the ordinary twitches. Pretreatment with a dose of d-tubocurarine which was about half of that having a minimal effect on the PTP gave a dose-dependent inhibition of the PTP by lidocain (50–100 mg/kg) in doses which otherwise were without effect. The inhibition of the PTP by lidocain or ouabain was not reversed by prostigmin. The single twitches could always be increased by prostigmin even if the PTP was totally inhibited.

Prostigmin inhibited PTP dose-dependently (ED_{50} = 1.0–1.5 μg/kg, n = 4, ED_{100} = 2.5 μg/kg, n = 4). The ordinary muscle twitches began to increase at a dose of 3–4 μg/kg.

Atropin was without any effect on the PTP in doses up to 50 mg/kg (Fig. 4). Haloperidol (10 mg/kg), mebumal (50 mg/kg) and diazepam (2 mg/kg) were without any effect on PTP.

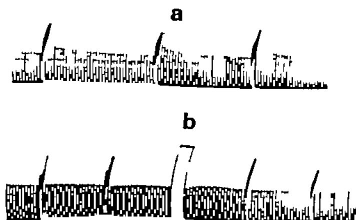


Fig. 3. Effect of ouabain on PTP. a) PTP after stimulation at 20 Hz for 5 s of untreated preparation. Tetanic stimulations at 30 Hz for 5 s and at 50 Hz for 20 s after infusion of 120 μg/kg ouabain. No PTP develops after stimulation at 20 Hz or at 50 Hz (for 20 s). The pretetanic contraction force is equivalent to 41 g. The 50 Hz stimulation for 20 s reached the maximum level of the Grass. Therefore the maximal contraction force can not be calculated.

Acta physiol. scand. 1978, 103, 165–172
From the Institute of Physiology University of Oslo, Norway

Rabbit lung plasma and erythrocyte volumes. Lung hematocrit in relation to total body hematocrit

By

P. Aarseth, B. A. Waaler and G. Nicolaysen

Received 5 December 1977

Abstract

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The total body hematocrit has been reported to be 85–90 % of packed cell volume (PCV) in several species. We have found similar values in rabbits. An "extra" plasma volume must exist somewhere in the vascular bed to explain this observation. We have looked for such an extra plasma volume in the pulmonary vasculature. The dynamic hematocrit was estimated in isolated, perfused rabbit lungs from distribution volumes for plasma and erythrocyte tracers. Estimation was also obtained from indicator-dilution curves using bolus-injections of such tracers avoiding their recirculation. It was thus possible to calculate mean transit times for the tracers from these dilution curves directly or applying monoexponential extrapolation on the first part of the downslope of the curves. The dynamic hematocrit of the lung vessels was about 95 % of perfusate PCV and there was no difference between the results obtained by the different methods. We concluded that in the rabbit only a very small part of the extra plasma volume is located in the lung vessels. The lung plasma volume is not underestimated by the indicator-dilution technique.

The plasma volume in a mammal is larger than estimated from total erythrocyte volume and packed cell volume (PCV, large vessel hematocrit). Thus the hematocrit as calculated from the measured plasma and erythrocyte volumes is about 90 % of PCV. Since blood in large vessels constitute a significant fraction of the total blood volume, the hematocrit in some organs must be lower than the total body hematocrit. However it is only in some minor parts of the circulation that dynamic hematocrits have been found to be smaller than that of the whole body.

The extra plasma volume has been explained in 2 different ways. When plasma volume is estimated, extravascular spaces will be included in organs with discontinuous capillary endothelium, as for instance the liver. The other explanation is based on the Fåhraeus-Lindqvist effect. This implies that with the axial streaming of erythrocytes, the small vessels will at any moment contain relatively less erythrocytes than plasma when compared to large vessels. Accordingly, in organs with a large proportion of small vessels, as for instance the lungs, the dynamic hematocrit should be rather low. In accordance with this

It should be noted that during physiological conditions, only end-plate receptors or receptors mediating PTP in fast mammalian muscles are active. PTP in fast muscles begin to develop at 1 Hz and is maximal after about 10 Hz, post tetanic repetition in slow muscle such as the soleus muscle, is only seen after stimulation at 400 Hz which is an unphysiological stimulation frequency. The present results show that PTP in the rat masseter mus. is mediated via the activation of nicotinic receptors which have different properties in comparison with the end plate receptors, they are "nondifferentiated" *i.e.* they are inhibited by both d-tubocurarine and hexamethonium. Since these nicotinic receptors are activated at physiological stimulation frequences (1 Hz) there evidently exists an additional synaptic mechanism in neuromuscular transmission in fast mammalian muscles. The possibility exists that activation of presynaptic nicotinic receptors are of importance for stimulation above 1 Hz in rat masseter muscle.

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Aarseth (1971) using a direct method found a very low dynamic lung hematocrit in a On the other hand indicator-dilution studies on larger animals and man have not sho any large extra plasma volume in this organ (Rapaport *et al* 1956, Brigham, Wooler and Staub 1975) This discrepancy could however be due to a systematic error involved the monoexponential extrapolation used on the indicator concentration curve in these la studies. Thus plasma volume in the lungs might behave as a two-compartment syst

In order to obtain more information on this problem we have used the indicator-dilu technique in isolated perfused rabbit lungs. Mean transit time for plasma and erythrocyt could then be calculated using both monoexponential extrapolation from the first p of the down-slope, and from the complete indicator curve as it was found with recirculation. Any systematic error in the analysis of the plasma or erythrocyte-cur should then be detected The dynamic hematocrit calculated from the mean transit tim was compared with that found from the distribution volumes of plasma and erythrocyt tracers.

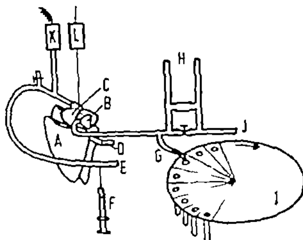
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Experiments were performed on 14 rabbits. In 3 rabbits (b.w.t. 2.8–3 1 kg) total plasma and erythroc volumes were estimated 11 rabbits (b.w.t. 2.8–3.5 kg) were used as donors for isolated perfused l preparations. I 3 of these lungs we measured the plasma and erythrocyte-volume from dilution valu of ^{125}I-serum albumin and ^{51}Cr-labelled erythrocytes. As a second method for erythrocyte volume det mination we lso measured the hemoglobin content in of these lungs. I the remaining 8 lungs performed 23 indicator dilution tests, by simultaneous bolus injections of ^{125}I-serum albumin and ^{51}C erythrocytes.

Total blood volume and total body hematocrit 3 ml rabbit erythrocytes were incubated with 0.3 µ $Na_2{}^{51}CrO_4$ and washed 4 times in heparinized saline (A rseth 1970). The labelled erythrocytes were su pended in saline containing 15 µCi ^{125}I human serum albumin (IFA, Kjeller Norway). The 3 rabbits we anesthetized by i v injections of alphaxolon and lphadolon-acetat (Alfathesin® Glaxo), and cathete were inserted in a femoral vein and in a artery About 70 min after the animals had recovered fr anesthesia a weighed amount of the isotope-mixture was given i.v Arterial blood samples ere taken 12 and 18 min thereafter PCV was found by centrifugation in an International microcapillary centrifu (Model MB), and the radioactivity in the blood samples nd in a dil ted sample of the isotope mixtu (1 250) were counted in channel Auto-gamma scintillation spectrometer (Model 220, Packa Instruments Co.). From the 3 observations of albumin distribution volume btained the plasma vol in each animal was estimated from extrapolation to zero time The mean of the 3 alues for erythroc volume was used a measure for the erythrocyte volume. Total body hematocrit as calculated erythrocyte volume/erythrocyte volume + plasma volume

Perfusion f the isolated lungs with homologous blood was carried out as described by Hauge, Lu and Waaler (1966) and the system is schematically shown in Fig. 1 The blood was pumped at consta flow from a reservoir kept av 37°C using a Harvard roller pump. The flow ranged bet een 139–320 ml m in individual experiments, djusted to give a pulmonary rterial pressure of bout 20–25 mmHg. Papaveri was added to the perfusat (3 mg/100 ml) to prevent vasoconstriction. An electromagnetic flow-pro (Nycotron) was connected to the inlet cannula, as was also a pressure transducer (Statham P23De). Th flow and inlet pressure were continuously recorded. The left trial pressure was kept at 5 mmHg through the expt The weight of the preparat on was continuously recorded (Hewlett Packard force transduc FTA 100-1). Positive pressure ventilation was performed with Starling pump, the inflation pressure w kept at 7 cm H_2O

Dilution volumes for ^{125}I-albumin and ^{51}Cr-erythrocytes in the lung I these expts. the volume of t circulating blood was kept as small as possible, bout 150 ml. Silk snares were placed around the pulmona artery and left atrial appendage. When perfusion pressure and preparation weight had stabilized, ^{51}C erythrocytes and ^{125}I-albumin were added t the reservoir d 8 min later the snares were sudde closed. The lungs were then immersed in liquid nitrogen, and the lung tissue was removed, cru homogenized. The hemoglobin content in the lung tissue was estimated by the the



g. 1 Schematical drawing of the perfusion system. The lungs (A) were perfused through cannula erted through the right ventricle (C) into the pulmonary artery. Inflow pressure was recorded from ssure transducer (K). The outflow from the left atrium (B) was diverted through a ladder (H) as to ntrol the pressure at the sampling site. The lungs were ventilated by positive pressure using pump nnected to the trachea (D). The lung weight was continuously recorded with force transducer (L). lus injections were performed with syringe-pistol (F) into the pulmonary arterial tube (E). A constant action of the outflow was diverted (through G) to the split fraction sampling device (I).

al. (1965). The plasma and erythrocyte volumes in the lung were calculated from the radioactivity in the sues and in samples of the perfusate. The erythrocyte content of the lung was also calculated from the hemoglobin content of the tissue and in the perfusate.

Indicator-dilution tests. Bolus injection of ^{125}I-albumin and ^{51}Cr-erythrocytes into the arterial cannula and continuous sampling from the outlet were carried out. PCV of the injectate was adjusted to be close to that of the perfusate. A syringe pistol giving a constant volume at constant speed was used for the injections. The sampling device described by Nicolaysen (1971) allowed split fractions to be sampled from the outflow every 0.67 or 1.16 s. Only part of the outflow was sampled, the remaining was discarded and thus recirculation of tracers did not occur. Flow through the system at the time of sampling was taken as the mean volume per unit time of collected outflow immediately before and after the bolus injection. Flow through the sampling catheter was found to be steady.

Since the flow was constant, the volume of the individual samples was nearly the same. The total ^{125}I- and ^{51}Cr-activity in each sample were counted. From these data, mean transit times for plasma and erythrocytes were calculated.

The following expts. were performed: Bolus injections of ^{125}I-albumin were given in the perfusion system with no lung in the circuit, and the volume of the tubings were calculated from flow and mean transit time for the tracer. Simultaneous bolus injections of ^{125}I-albumin and ^{51}Cr-erythrocytes were also given, and the mean transit time for plasma and erythrocytes through the tubings were compared.

Bolus injections were performed with lung in the perfusion circuit. In each expt. 2–4 injections were given. In 2 expts. with 8 injections, the transit time for plasma and erythrocytes through the tubings and through the sampling catheter were known. In these expts. then the mean transit times through the lung vessel proper and thus their volumes could be calculated.

The dilution curves from these 2 expts. and from 15 similar tests in 6 other expts. were analyzed in 3 different ways.

$$\bar{t} = \sum_{i=0} (cpm_i \cdot t_i) / \sum_{i=0} cpm_i \qquad (1)$$

here $\bar{t}$ is the mean transit time, cpm_i is the radioactivity in sample, t_i the time from injection to that particular sample, number of samples in the series was usually about 35.

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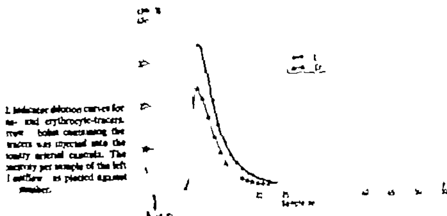
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1 Indicator dilution curves for ... and erythrocyte-tracers. ... bolus containing the tracers was injected into the ... arterial cannula. The activity per sample of the left ... outflow is plotted against ... number.

and 0.7 ml, respectively and the dynamic hematocrits in % of PCV of perfusate were 3 94.6 and 95.0. The last lung in this series was perfused with pulsatile flow. This made difference as regards plasma excess or pulmonary blood volume (Table II).

The volume of the tubings in the perfusion system was found to be 20.15 ml (median, range 7-20 3). The ratio between the transit time for ^{131}I-albumin and that of ^{51}Cr-erythrocytes these tubings was estimated in 8 bolus-injections. The median value was 1.004 (range 955-1 040).

Fig. 2 shows a set of indicator-dilution curves obtained in one of the perfused lungs. The mean transit times for plasma and erythrocytes were calculated from 8 injections, and the ... were corrected for delay in the tubings and in the sampling catheter. The results are shown in Table III. The median value for dynamic hematocrit was 93.8 % of perfusate PCV (range 90.5-95.0) and thus identical with that found with the direct method. The plasma excess in the lungs were 1.25–2.0 ml (median value 1.55 ml). The data from the 23 bolus injections of ^{131}I-albumin and ^{51}Cr-erythrocytes were analyzed in different ways as

TABLE III. Mean transit times for plasma and erythrocytes in perfused rabbit lungs. Plasma- and erythrocyte-volumes were calculated from flow, PCV of perfusate and the transit times. Excess plasma ... the difference between this plasma volume and that calculated from erythrocyte volume and PCV ... also calculated in ... of actual lung blood volume.

	Flow ml, min	PCV of perfusate	$\bar{t}_{plasma}$	$\bar{t}_{eryth.}$	Lung plasma ml	Lung eryth. ml	Excess plasma ml	Excess plasma %
Exp. 16-9								
1	240	34.7	7.5	6.8	19.6	9.4	1.9	6.5
2	244	35.3	7.3	6.75	19.3	9.7	1.6	5.5
3	246.5	35.8	7.3	6.7	19.2	9.9	1.5	5.2
Exp. 23-9								
1	208	28.3	6.4	5.75	15.8	5.75	1.25	5.8
2	198	30.3	6.6	5.7	15.1	5.7	2.0	9.6
3	196	30.7	5.9	5.3	13.4	5.3	1.4	7.5

TABLE I. Distribution volumes for ^{125}I human serum albumin and ^{51}Cr-labelled rabbit erythrocytes in rabbits. Plasma excess is the difference between the measured plasma volume and that calculated from erythrocyte volume and PCV. It is also given as % of total blood volume.

	Rabbit no		
	1	2	3
Body weight, kg	3.1	3.0	2.8
Plasma volume, ml/kg b.wt	32.8	34.2	33.4
Erythrocyte volume, ml/kg b.wt.	15.0	15.5	14.0
PCV	35.5	34.6	34.3
Plasma excess, ml/kg b.wt.	5.3	4.7	6.5
Plasma excess, %	11.1	9.5	13.7

) From the 5 first samples on the downslope of the indicator curve, the best fit (least square deviation) monoexponential curve was found. The measured values for cpm_i were replaced with values taken from this monoexponential curve and $\bar{t}$ was calculated as for 1).

3) All samples on the downslope part were used to find the best fit bi-exponential curve (least square deviation). cpm_i-values were read from this curve and used in the formula given in 1). The calculation was also extended to infinity. The best fit analysis were done on a Nord 10 computer (Norsk Data).

Results

The results for body plasma and erythrocyte volumes are given in Table I. The estimated plasma volumes were 14, 16 and 18 ml respectively larger than could be expected from the measured erythrocyte volumes and PCV. Total body hematocrits thus were 88.7, 90.8 and 85.5% of PCV respectively in the 3 animals.

Table II shows the results from the 3 lungs in which plasma and erythrocyte volumes were determined from the distribution volumes of tracers. The erythrocyte volume was determined in all 3 from the ^{51}Cr-erythrocyte dilution volume, in two of them also from the hemoglobin content of the lung tissue. As is seen in Table II the two methods for determination of erythrocyte volume gave nearly identical results. The plasma excess was 1[illegible]

TABLE II. Plasma volume as estimated in isolated perfused rabbit lungs from distribution volumes of ^{125}I human serum albumin. Erythrocyte volumes are found from the lung content of ^{51}Cr-labelled erythrocytes or hemoglobin. Plasma excess is calculated as difference between measured plasma volume and that calculated from PCV and erythrocyte volume.

	Rabbit no.		
	4	5	6
Body weight of donor rabbit, kg	3.15	3.15	3.40
Lung plasma volume, ml	9.29	8.6	10.08
Lung erythrocyte volume, ml			
^{51}Cr-erythrocytes	4.50	4.13	4.82
Hemoglobin	5.19	4.49	
PCV of perfusate	35	35.8	34
Plasma excess in lung, ml	1.0	0.7	0.7
Plasma excess in lung, %	7.3	5.8	4.7

volumes of plasma and erythrocyte tracers in rapidly frozen animals. The dynamic atocrit found has almost invariably been significantly smaller than the total body hematocrit (Aarseth 1971). This is in contrast to the results obtained in larger animals with more indirect methods referred to above. This discrepancy could be explained if there is a second plasma compartment in the lungs, undetected by the indicator-dilution techniques when monoexponential extrapolation is used to circumvent the recirculation. In the present expts. on isolated, perfused lungs, we were able to avoid the monoexponential extrapolation. It turned out that mean transit times calculated from extrapolated curves were shorter than the observed transit times. Most of this difference must be due to the perfusion system as it also was found in model expts. with no lung in the circuit. It remained a small but insignificant difference which was slightly larger for the plasma than for the erythrocyte transit time, this would hardly make any difference as regards the pulmonary dynamic hematocrit.

The indicator dilution curves thus did not have a monoexponential downslope. Even in a two-compartment system, however, the indicator dilution curve will end in a monoexponential tail (Larsen and Sejrsen 1971). By ending the sampling before this tail is reached, the mean transit time calculated from extrapolation would be seriously underestimated (Larsen and Sejrsen 1971). In the present experiments, the median concentration of last sample was 0.3 of peak concentration (96.5% interval 0.25–0.6). For one curve, extrapolation was done from a sample which indicator concentration was 6% of peak concentration. The mean transit time calculated from this extrapolated curve was negligibly smaller (3%) than the one calculated from the observed points. We therefore suggest that we have reached the monoexponential tail in our expts.

The mean transit times calculated from the observed points were identical with those obtained by integration of a biexponential curve fitted to the observed points. The second exponential probably will describe the tail. By integration to infinity of this biexponential curve, the mean transit times increased by only 2.76% and 2.07% for plasma and erythrocytes, respectively. It can be concluded that monoexponential extrapolation from the first part of the downslope does not to any large extent underestimate mean transit time for plasma as compared to that of erythrocytes.

The vascular volume seen by the indicator dilution technique includes the pulmonary artery and left atrium, and will thus be larger than the distribution volume within the lung tissue proper (Table II and III). The latter volume was determined from both ^{51}Cr-erythrocytes and hemoglobin in the homogenized lung, and the two methods differed by only 8 and 13% in the 2 expts. This suggest that the labelled erythrocytes are fairly well distributed, and thus also the labelled albumin.

In conclusion, if monoexponential extrapolation of indicator-dilution curves gives wrong estimates of transit times, then this error will be the same for plasma and erythrocytes. In our isolated lungs, there seemed to be no plasma compartment unseen by the indicator dilution tests. We do not know therefore in which vascular compartment the extra plasma volume is located.

We would like to thank research fellow Gunnar Sydnes for invaluable help with the data-processing. We also thank Karin Langerud for skilful technical assistance.

described in Methods. For each curve the mean transit time was calculated from observed concentrations of indicators throughout the sampling period. This value was to 100 %. Using monoexponential extrapolation from the first 5 samples on the downslope we found. The median value for the mean transit time of erythrocytes was 90.01 % (95 % confidence interval 87.90–92.32) while for plasma it was 88.88 % (86.82–90.41). The difference between the mean transit times obtained from the observed curve and from monoexponential extrapolations were also seen in the model experiments with no lung in the circuit (median values 91.57 and 91.64 % for erythrocytes and plasma respectively).

The analysis in which all the points on the downslope were fitted to a biexponential curve and mean transit times calculated from the integral up to the time of the last sample gave the following results. The median value for plasma was 99.89 % (99.63–100.08) while that for erythrocytes was 99.88 % (99.51–100.32). These biexponential curves were also integrated to infinity, the mean transit times calculated then were 102.76 % (101.51–104.76) and 102.[illegible] (100.74–105.07) for plasma and erythrocytes, respectively.

Discussion

The median value for total blood volume in 3 rabbits was 47.8 ml/kg b.wt., which is in the lower normal range (Little 1970). The total body hematocrit was between 85–90 % of PCV, the same order of magnitude is found in man and dog (Gregersen and Rawson 1959), cat (Aarseth and Bø 1972) and rat (Aarseth 1970).

The dynamic hematocrit in different tissues can be calculated from dilution curves using both plasma and erythrocyte tracers. With Bradley's equilibration method, Larsen and Lassen (1964) and Larsen (1966, 68) measured the dynamic hematocrit in the cerebral vessels, in the lungs and in the lower extremety. In all these organs they found dynamic hematocrits which were larger than total body hematocrit. Similar results have been obtained by Crane *et al.* (1960) who applied the Stewart Hamilton technique on the same organs. Rapaport *et al.* (1956) and Brigham, Woolverton and Staub (1975) used the method on lungs in dogs and sheep and reached the same conclusion. Larsen, Tygstrup and Winkler (1963) applied Bradleys method on the splanchnic circulation and measured a dynamic hematocrit in this area that equelled total body hematocrit. In patients with portacaval anastomosis they were able to study the liver vessels separately and revealed a dynamic hematocrit of only 86 % of total body hematocrit (Larsen, Winkler and Tygstrup 1963). Aarseth, Aarseth and Bergan (1976) measured plasma and erythrocyte distribution volumes in rat liver. Only in animals with bile duct occlusion was the liver dynamic hematocrit smaller than the total body hematocrit. In control animals it was slightly larger. Studies on the renal circulation with the Stewart Hamilton method have revealed a dynamic hematocrit that is similar to that of the total body hematocrit (Chinard, Enns and Nolan 1964), while direct estimates of distribution volumes have unveiled a slightly smaller hematocrit in this organ (Rasmussen 1973). Thus so far the excess plasma volume known to exist has not been found in any of the organs studied.

In our laboratory the dynamic hematocrit of the rat lung has been evaluated from distribution

Acta physiol. scand. 1978. 103 173–179
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Maintenance of lactose secretion during acute insulin deficiency in lactating goats

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Received 10 December 1977

Abstract

HOVE, K. *Maintenance of lactose secretion during acute insulin deficiency in lactating goats.* Acta physiol. Scand. 1978 *103* 173–179

Induction of alloxan diabetes in 5 lactating goats resulted in reduced milk yields in 3 of the animals, while yield was unchanged in two. After treatment of the diabetic goats with insulin for 4–5 days—the first 4 intravenously—lactose secretion returned to the control values before alloxan administration provided normoglycaemia developed. In experiments infusion of a large dose of insulin caused hypoglycaemia and 20–30 per cent reduction in lactose secretion rates. In the course of 1 h after withdrawal of the insulin infusion, potent signs of insulin deficiency developed as evidenced by steadily increasing plasma glucose concentrations. Nevertheless, lactose secretion continued at the same rate as during insulin infusion in the 4 h studied after discontinuation of the insulin infusion. In the goats where lactose secretion was reduced due to insulin-induced hypoglycaemia, lactose secretion returned to control values when following discontinuation of insulin infusion the plasma glucose concentrations increased into normal and diabetic ranges. It is concluded that during insulin deficiency of short term duration, mammary lactose secretion is maintained at normal rates. Since lactose is the major product of mammary glucose utilization, it is suggested that glucose uptake in the mammary gland was not reduced by short term insulin deficiency.

Milk secretion is a process which proceeds at a nearly constant rate throughout the day irrespective of changes in absorption of metabolites from the digestive tract and irrespective of diurnal variations in the plasma concentrations of hormones (e.g. insulin) regulating the disposal of energy substrates. In lactating ruminants a considerable amount of the available energy substrates is used for milk synthesis. Thus, the glucose uptake of the mammary gland accounts for 60–85 per cent of the glucose utilization in lactating dairy ruminants. Two thirds or more of the mammary uptake is converted to lactose (Annison and Linzell 1964, Bickerstaffe *et al.* 1974). An important question therefore arises. Is glucose uptake by mammary glands influenced by plasma insulin concentrations in a similar way as glucose uptake in peripheral insulin-sensitive tissues? Since the synthesis of lactose by the mammary gland accounts for the major part of the mammary glucose uptake in dairy ruminants, any major changes in glucose uptake might also result in altered rates of lactose secretion.

The question can only be answered partly. Insulin is in fact essential for the maintenance of secretory activity in mammary tissue both in rodents (Walters and McLean 1968, Martin

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ith heparinized saline (5 IU/ml) between samplings. T pairs of arterial and venous blood samples taken every hour 20 and 50 min after each oxytocin injection.

and procedures

he plasma was measured spectrophotometrically by glucose oxidase method (Boehringer reagents). measurements were run in duplicate and the standard deviation of duplicates in the normal plasma ie range (60–160 mg/100 ml) as 1 mg 100 ml and in the diabetic range (200–300 mg 100 ml) between 9.4 mg/100 ml.

ma acetoacetate as measured spectrophotometrically by an automated modification of the nitro-ide reaction in dialysate of plasma (Blom and Hulse 1975).

ma free fatty acids as measured by the radio-active nickel binding method of Ho (1970) ith modifications.

ma insulin as measured by radioimmunoassay using dextran coated charcoal to separate bound ee insulin (Porzonski and Porzonski 1969). Bovine insulin as used both as standard and for the ation of anti-insulin antibodies. An antibody concentration giving maximum sensitivity in the he range of insulin concentrations was chosen. The samples were measured in duplicate. The ariation icient varied between 17 in the lower range (0.2–0.6 ng/ml) and 7 in the higher range of insulin ntrations. The lower limit of detection usually bout 0.1 ng insulin per ml of plasma.

lk lactose as measured spectrophotometrically as glucose (by the glucose oxidase reaction) after tose hydrolysis by 1.3 U per 5 ml of E. coli β-galactosidase (Boehringer). The milk first diluted the proteins precipitated (Somogyi 1945). A sample containing 50–100 µg lactose as incubated enzyme and 5 ml of glucose oxidase reagent (Boehringer) at 37 C for 45 min. Absorbances were on Beckman DB spectrophotometer at 620 nm. Analytical grade lactose was used as standard. linear relationship as obtained between absorbance and lactose concentration within the actual range. milk samples ere analysed in duplicate. The coefficient of variation on duplicates as between 2 and

Results

betic changes in the metabolism were evident 48 h after alloxan injection. Low plasma lin and high plasma glucose concentrations (Table I) were accompanied by increased centrations of plasma free fatty acids and acetoacetate (from 0.24 to 0.55 and from 0.04 0.19 mM on the average, respectively). At this time of the expt. lowered milk yields were ained in 3 of the goats (from 20–75 per cent of the control yields), while the yields in the 2 others were essentially unchanged.

) I the 4 expts. where normal plasma glucose concentrations were obtained after the ulin infusion the hourly lactose yields were approximately identical to those observed in control experiments immediately before alloxan administration (Table II).

1) When the infusion was discontinued a sharp rise in blood glucose levels was observed all goats. During the 4 h observation period, plasma glucose concentrations of between

ble I Changes from control to alloxan diabetic state. Control. Average and S.D. of plasma concentrations in 10 arterial samples taken during 5 h period bef measured pre-alloxan lactose secretion. Alloxan average and S.D. of 5 arterial samples taken at hourly intervals starting 48 h after alloxan administration.

at	Insulin ng/ml		Glucose mg/100 ml	
	Control	Alloxan	Control	Alloxan
	0.32 ± 0.12	0.27 ± 0.03	65.9 ± 4.2	295 ± 6
	2.00 ± 0.30	0.15 ± 0.03	69.1 ± 3.5	254 ± 15
	0.67 ± 0.20	0.25 ± 0.08	75.8 ± 3.1	265 ± 21
	0.92 ± 0.17	0.25 ± 0.02	80.0 ± 6.1	305 ± 7
	0.58 ± 0.06	0.10 ± 0.01	65.6 ± 2.0	308 ± 12

and Baldwin 1971 *a b*) and goats (Nowak and Dzialoszynski 1967) Lack of insulin in rat for 36–48 h or more reduces milk secretion and enzymatic activity in the mammary gl. Alloxan diabetes of some weeks duration causes complete and irreversible loss of secr. activity in lactating goats These changes are probably related to the deterioration of metabolic machinery of the mammary cells, and do not give information as to the pos regulatory role of insulin in mammary carbohydrate uptake and secretion. While the or short term effects of insulin deficiency in the rat include reduced glucose oxidation, fat lactose and casein synthesis (Baldwin and Louis 1975) comparable information ruminants is lacking. Diabetes mellitus is a very rare disease in ruminants and has not been reported during lactation (Phillips *et al* 1971) Therefore, to study the short-term effect insulin deficiency lactating goats were made diabetic by administration of alloxan. Mam. lactose secretion was measured in two situations After a 24 h i v infusion of insulin to normalize the metabolism, and subsequently for four hours after the infusion was discontinued. During this last period marked metabolic signs of insulin deficiency develop

Methods

Animals

5 adult goats weighi g 30–45 kg were used for the present study The animals were in second to sixth m of lactatio and yielded from 0.9 –.0 kg milk daily The goats were housed indoors in individual p during the experiments and fed hay and a pelleted concentrate ration *ad lib*. Food was given twice daily about 8 a.m. and 3 p.m., nd milki g was performed in connection with the meals. Feeding and m were carried out as usual on days when experiments were to be performed. The goats were allowed to undisturbed for ½ 1 h before the experiments started. Some weeks prior to the experiments or artery was exteriorized into a skin-covered loop to facilitate the sampling of arterial blood.

Experimental design

Alloxan diabetes was established by i. i jection of D-alloxan at a dose which has previously pr effective in goats and sheep (50 mg/kg b wt., Nowak nd Dzialoszynski 1967 Flisell *et al* 1969) Blood sampled 48 h after lloxan administration and plasma concentrations of insulin, glucose free fatty acids acetoacetate were measured in order to erify the presence of a diabetic cond tion. The animals were stabilized with insulin for 4–5 days. Lactose secretion was measured in two experimental settings—in treated alloxan diabetes and insulin deficiency—and compared with values obtained one to [illegible] to allox n administration.

1) *Insulin treated alloxan diabetes.* Insulin treatment was started 48 h after allox administration. In (porcine) was injected subcutaneously for 3–4 days and subsequently infused i. for 4 h. During Infusion insulin diluted with saline was gi en through jugular catheter at a rate of 1 7 to .. [illegible] (4 6 ml/h) the aim being t achieve a normal plasma glucose level. After at least 20 h (usually 4 [illegible] insuli treatment lactose secretion was measured fo 2 h. I 3 goats approximately normal plasma glu levels were observed while in 2 hypoglycemic glucose values (35–45 mg/100 ml) were obtained at the of the insulin infusion. In one of the latter a lower dose of insulin was infused 2 days later On this occ normoglycemia was maintained throughout the last part of the nfusion. Consequently 4 expts. w normal and two with hypoglycemic glucose levels are reported.

2) *Insulin deficiency* The infusion of insulin was discontinued after 2 h of milk and blood sampl Changes in plasma glucose concentration and lactose secretion were then studied for 4 h during the development of a diabetic condition.

Milking I travenous injection of 100 mU of synthetic oxytocin as gi en to evoke milk ejection (Lind 1960). 5 min elapsed from oxytocin administration to the completion of milking. The goats ere milked by hand and the volumes from the two udder halves wer measured separately

Blood sampling Catheters for blood sampling (o.d 1.0 mm) were inserted i t the carotid artery [illegible] under local nesthesia. Plasma was separated, frozen nd kept t – 20°C until analysed. The catheters we

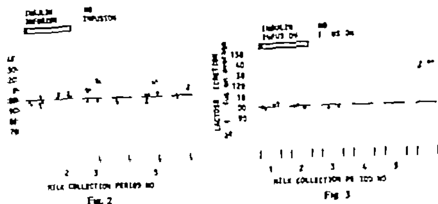


Fig. 2 Lactose secretion during and after the infusion of insulin to 4 alloxan diabetic goats. Lactose secretion in per cent of the average secretion for each gland during the 2 h infusion period. R and L: Right and left gland respectively. Individual symbols.

Fig. 3. Lactose secretion during and after the infusion of insulin to 2 alloxan diabetic goats. Due to the use of too large dose of insulin hypoglycemia developed at the end of the infusion (see Fig. 1). Legend as in Fig. 2.

ose secretion varied somewhat, but no significant changes were observed (Fig. 2). In the 2 expts. with hypoglycemic goats lactose secretion was increased by 30–40 per cent following cessation of insulin infusion (Fig. 3). During the infusion period lactose secretion was 20–30 per cent lower than that recorded in the control expt before alloxan administration (Table II). Thus the increase observed after insulin withdrawal in these goats actually seems to have been a 'normalisation' of lactose yield which was depressed by hypoglycemia during the insulin infusions.

Discussion

The changes in plasma metabolite concentrations after alloxan administration were comparable with those observed in sheep by Jarrett *et al.* (1974), and indicate that the amounts of insulin secreted by the pancreas 48 h after alloxan administration were insufficient to maintain normal fat and carbohydrate metabolism. Insulin was given to the alloxan diabetic goats for 4–5 days to allow time for a normalization of the diabetic metabolism before the lactose secretion measurements were carried out. The finding that lactose was secreted at approximately the same rate as in the control situation at the end of the 24 h insulin infusion, indicates normal metabolism at this time of the experiment.

After discontinuation of the insulin infusion plasma insulin concentrations declined rapidly. But the insulin deficiency was not associated with reduced lactose secretion despite the fact that patent signs of a diabetic metabolism developed during the last 2–3 h of the observation period. The rise in lactose yields (by 30–40 %) after the cessation of insulin infusions in the 2 expts. with hypoglycemic animals further substantiates this. In spite of the insulin deficiency lactose secretion in the 2 hypoglycemic goats increased to the levels observed before the alloxan injections when plasma glucose returned to normal or hyperglycemic levels.

The importance of insulin as major regulator of energy metabolism in ruminants has

TABLE II Lactose secretion rate in the control experiment before alloxan administration and the restoration of the alloxan diabetes by a 4 h insulin infusion. Before: average and S.D. of 3 one hour periods. Insulin treated diabetes: average of one hour periods at the end of a 4 h infusion. R and L: Right and left mammary gland.

Goat	Before alloxan		Insulin treated diabetes	
	R	L	R	L
B	20.1±1.7	19.9±0.9	21.6	22.6
C	3_.1±3.2	34.8±_.5	34.1	33.0
			23.9	26.7
D	25.9±2.0	30.1±3.2	28.9	34.0
E	23._±1.1	_2.5±0.8	18.0	18._
M	20.3±1.2	17.9±_.0	16	19.7

Hypoglycemia due to the infusion of a too large dose of insulin.

300 and 500 mg/100 ml were reached in most of the animals (Fig. 1). Plasma insulin concentrations decreased within 20 min after the discontinuation of the insulin infusion (Fig. Insufficient time elapsed so as to allow insulin to fall to the low levels seen in untreated alloxan diabetes (Table I). The high plasma glucose concentrations indicate a diabetic metabolism, however. In the 4 goats with initially normal plasma glucose concentrat

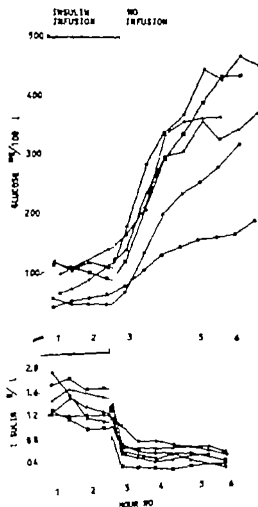


Fig. 1 Plasma glucose and insulin concentrations during and after the 4 h infusion of insulin in alloxan-diabetic goats. 6 different curves are shown. The symbols O—O and ▲—▲ refer to the same goat during infusions which gave hypoglycemic and normal plasma glucose concentrations respectively.

[illegible] technical assistance of M. Per Maen is acknowledged. The study [illegible] supported by the Nor[illegible] Agricultural Research Council.

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been definitely settled (Review Basset 1975) Considerable diurnal fluctuations in pl
insulin concentrations have been observed in various physiological states (Lactation H
and Blom 1973 Hart *et al* 1975 Pregnancy Hove and Blom 1976) Periods with h
insulin concentrations are associated with feeding, while the 24 h minima coincide w
periods with low absorption of metabolites from the gastrointestinal tract. The pre
results demonstrate that the mammary secretion of lactose was in fact unaffected by s
term changes in the plasma concentrations of insulin to an extent which profoundly aff
the carbohydrate metabolism of other tissues This constancy in lactose secretion coul
explained by the existence of an insulin-independent glucose uptake mechanism in mam
tissue The mammary cells would then continue to utilize glucose at their normal r
irrespective of the rapidly vanishing plasma insulin concentrations. The arguments for
presence of an insulin-independant uptake mechanism for glucose in the udder rests o
the assumption that the half life of insulin action on the transport mechanism is of
same magnitude as in other tissues, *i.e* well below the 4 h studied in the present exp T
experiments referred to earlier involving long term insulin deficiency (Nowak and Dzi
zynski 1967 Martin and Baldwin 1971 *a* *b*) demonstrate that a continuous secretion
insulin is a prerequisite for milk secretion The time required to obtain reductions in la
and milk production in insulin deficient animals (usually in the order of days) is r
longer than that required to change the rates of carbohydrate transport in insulin sens
tissues. Thus, although alternatively insulin might be an active regulator of mam
glucose uptake, most available evidence seems to favour an explanation involving
insulin independent glucose uptake mechanism in the mammary gland From experim
in rats Martin and Baldwin (1971 b) suggested that the primary action of insulin on mam
cells was to regulate intracellular processes, and not the transport of nutrients. Fur
Hove (to be published) in another series of experiments could not find any effect of in
on mammary glucose uptake and lactose secretion rate in goat mammary glands rece
blood with artificially increased arterial plasma insulin concentrations.

The pronounced rise in arterial glucose concentrations after insulin withdrawal cou
the effect of changes in both hepatic and extrahepatic carbohydrate metabolism The
of ruminants does not seem to take up significant amounts of glucose (Review Ba
et al 1969) but the rate of glucose release is strongly influenced by insulin (West
Passey 1967) and by glucagon (Basset 1971 Clark *et al* 1976) Although plasma gluc
concentrations were not measured in the present investigation, reduced insulin/gluc
ratios favouring hepatic glucose release would be the consequence of insulin withdr
Reduced glucose consumption in peripheral tissues during insulin withdrawal could
contribute to the glucose rise, although the quantitative importance seems unclear a
present time (Cahill 1971 Jarret *et al* 1974).

In conclusion, the present study demonstrates that in the dairy goat a normal la
secretion can be maintained during periods of insulin deficiency which are sufficiently
to cause great changes in the carbohydrate metabolism of the animal In contrast to
a more prolonged lack of insulin (for days or weeks) will invariably lead to drastic
irreversible decreases in milk production as demonstrated in the goat by Nowak
Dzialoszynski (1967)

thus evident that the epaxial muscles form an integral part of the locomotor mechan- yet few data are available about their physiological properties and reflex interactions. nt neurophysiological investigations of the longissimus and multifidi muscles in the lumbar back region (Carlson and Lindquist 1976) have shown that these muscles, like scors in the leg, are tonically active in decerebrate preparations and that their activity is enced by exteroceptive stimulation applied to certain skin areas on the trunk and on imbs. The localization of skin areas from which facilitation or inhibition of motor unit ity could be evoked prompted speculations about the contribution of back muscles in drawal reactions of the animal. However for further analyses of extero- and pro- ceptive reflex patterns in the lumbar back muscles as well as for studies of their con- tion properties it proved necessary to gather additional information about the mor logy and innervation of the muscles. Such observations will be presented in the first of this paper The structural organization of the lumbodorsal fasciae will also be de bed The second section of the paper deals with the contraction properties of the two ral muscles longissimus and iliocostalis and the medially located multifidi and inter ales.

histochemical investigation of these muscles will be presented in a subsequent paper rlson 1978).

Material and methods

Morphological observations on the lumbar back muscles were made in about 25 cats also utilized for er purposes. In four cats the individual muscles ere dissected free and weighed. Cross sections of the lbar region were obtained by sawing one embalmed and one frozen preparation. The sections were ined at low magnification (6–16) and photographed The mobility of the lumbar spine was estimated in X-ray pictures of deeply anesthetized but otherwise intact animals. The cats were placed lying on one and manually held in different postures during the exposures.

The contraction properties of the medial muscles multifidi and interspinales and the lateral muscles gissimus and iliocostalis ere studied in 14 cats (2.8–5 4 kg) anesthetized by i.p. injections of Nembutal (–40 mg/kg b wt.). Due to the close anatomical relationship between the medial muscles as ell as be- en the lateral muscles the preparation of individual muscle attachments for recording of mechanical ponses liable to injure muscle fibers and to interfere ith the blood supply Another factor limiting possibilities of selective studies is that the various branches of the dorsal rami of the spinal nerves plying individual muscles generally run intramuscularly throughout their course. Hence, preparation these branches for indirect activation of the muscles might also cause some damage to them. With the perimental procedure used, as described below the conclusions drawn in the analyses of the contraction operties of the various muscles should however be reasonably safe

A midline skin incision was made extending from the upper thoracic to the sacral region and then the cial layers in the lumbar region and the latissimus dorsi were cut through bilaterally

From the multifidi and interspinales muscles at given level along the lumbar spine recordings ere ade by connecting the appropriate tendon to the recording instrument leaving the insertion onto the inous process intact (cf Fig 1) The longissimus and iliocostalis are separated from the iliac bone to- ether with small segment of the iliac crest which was attached for mechanical recording. I some ex iments the deep fascial layer (cf Fig 1 2) was connected for registration of contractions. Even though he direction of pull as adjusted so as to give maximal contractions the recordings did probably not epresent the maximal force of the muscles.

The muscles were indirectly activated by stimulation of their peripheral nerves or the corresponding ventral roots. The former procedure was applied to the multifidi and interspinales which both are supplied by medial branches of the dorsal rami. No attempt was made to separate and selectively stimulate the in- dividual nerves to these muscles. The nerve arising from given segment was dissected free for stimulation lateral of the superior articular process. For activation of the longissimus and iliocostalis ventral root stimulation as applied after laminectomy of the lumbar spine and transection of the dorsal roots. T

Acta physiol. scand. 1978, 103, 180–197

From the Department of Physiology II Karolinska Institutet, Stockholm, Sweden

Morphology and contraction properties of cat lumbar back muscles

By

HANS CARLSON

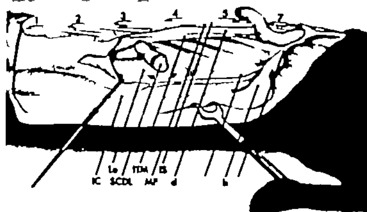
Received 13 December 1977

Abstract

CARLSON H *Morphology and contraction properties of cat lumbar back muscles.* Acta phys scand. 1978 *103* 180–197

The gross morphology innervation pattern and contraction properties of lumbar back muscles in the were investigated The medially located multifidi and interspinales are formed by short bundles connecting adjacent ertebrae. Laterally located bundles composing longissimus and iliocostalis at tached to the spinous processes by fascial layer and to the pelvic bone by a w ll developed intermuscular septum Different spinal segments innervate the various sections along the muscles in such a way the myotomes composing a muscle belly seem t be arranged after one another in a row The contraction of a maximal isometric twitch is 34 ms for multifid and interspinales and 29 ms for longissimus and iliocostalis. No large segmental variations of contraction times were found. The time course of twitch and a study of reflex co tractions in middle and lower sections of lateral muscles indicate the presence f a slowly contracting portion located to the longissimus. The initial muscle length is more decisive twitch amplitude of medial than of lateral muscles. In medial muscles summation of individual twitch starts at a stimulus frequency of 10–15 Hz and apparent fusion occurs at about 50 Hz. For lateral muscles corresponding values are 15–20 Hz and about 70 Hz. The contractile tension declines markedly muscles during a 10 min period of stimulatio t 5 Hz.

While a considerable amount of information is available about the functional organization of mammalian limb muscles, very little attention has been paid to the muscles primarily responsible for movements and posture of the vertebral column. The significance of the epaxial muscles for locomotion has been considered in studies concerning the mechanics of the tetrapod skeleton (Gray 1944 1968) and of the vertebral column (Slijper 1946) and in an analysis of the motions of the running horse and the cheetah (Hildebrand 1959). From these works it appears that in lower tetrapods the propulsive drive may have its origin in the vertebral muscles whereas in mammals these muscles provide the trunk with adequate rigidity during locomotion. In addition, certain animals, *e.g.* the cat, display marked flexions and extensions of the axial skeleton during running (Goslow Reinking and Stuart 1973). These movements, in part executed by the epaxial muscles, are coordinated with limb movements so that an increase of the stride is achieved



1. Dorsolateral view of muscles in the cat' lumbar back region. Lo, longissimus, IC, iliocostalis, SCDL, sacrocaudalis dorsalis lateralis (cut and partly freed from Lo); ITM, intertransversarii mediales, MF, multifidi, IS interspinales 1 5 and 7 spinous processes of lumbar vertebrae a, iliac crest, b and c as covering dorsal muscles, cut and retracted to show structure and fiber directions of underlying cles, d, fascia covering multifidi muscles, cut along dorsolateral spect of muscles.

the total epaxial muscle mass in the region while the multifidi represent around 10 ... sacrocaudalis dorsalis lateralis about 8 and the intertransversarii mediales together h the interspinales only a few percent.

Before giving a more detailed description of the muscles it seems pertinent to consider the ganization of the various connective tissue structures in the lumbar region.

T o separate fascial layers, attached to the iliac crest, cover the dorsolateral aspect of e back muscles (cut and retracted in Fig. 1). Dorsally they are attached to the spinous ocesses and the supraspinous l gaments. The thin superficial sheet covers all the muscles d inserts into the transverse processes and the intertransverse ligaments (*b* in Fig. 1 2) he inner layer which is more prominent, covers the medial muscles and the dorsolateral rface of the longissimus and then enters the lateral muscle mass (in Fig. 1 2).

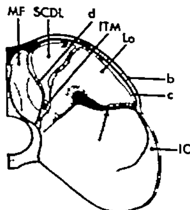


Fig 2 Cross section at level of 6th lumbar vertebra. Fascia and muscles marked as in Fig 1 Darker dotted area of Lo shows localization of muscle tissue appearing red in color Arrow indicates approximate position of fusion between lateral and medial connective tissue components of inter muscular septum.

avoid mechanical disturbances from other muscles the ventral rami of the spinal nerves were ... periments applying ventral root stimulation. For elicitation of reflex contractions of the ... iliocostalis dorsal roots were stimulated.

The animal were fixed in a rigid frame by tapered rods inserted from both sides into the ... by a clamp holding spinous processes in the thoracic region. Unilateral fixation of ... processes ensured stability of the spine during muscle activation. The skin was sewn onto the frame ... a pool which was filled with liquid paraffin. The temperature in the pool was kept at 36-38 C by ... red heating lamp. The appropriate ends of the cut nerves or of the roots were stimulated with ... silver-silver chloride wire electrodes. In some experiments, when observing the contractions of the ... simus and iliocostalis muscles, steel needle electrodes insulated to their tips were inserted into the ... bellies in order to stimulate individual nerve branches supplying the different muscles. Stimuli ... 0.3 ms duration were delivered by a Grass S4 stimulator.

Near-isometric muscle contractions were recorded with a Grass FT 03 or FT 10 transducer (... frequency 500 and 1 000 Hz respectively) connected to the muscles by short lengths (30-60 mm) of ... material (Mersilene® no 1). The transducer was connected either to a cathode follower and a Grass ... amplifier or, when studying the development of muscle fatigue, to an inkwriter (Grass polygraph). Muscle action potentials were recorded with fine copper wire electrodes insulated except at the ... inserted into the muscles through a hypodermic needle or with DISA needle electrodes. The ... displayed on a Tektronix 502 dual beam oscilloscope and photographed with a Grass C4 camera.

The development of muscle fatigue was studied systematically during 10 min periods of stimulation ... 5 Hz since the further decrease in tension was small with longer period. The frequency of 5 Hz ... twitch contractions, thus the blood pumping action of the muscles is preserved and the interference ... the blood supply should be minimal (cf. Edström and Lindquist 1973).

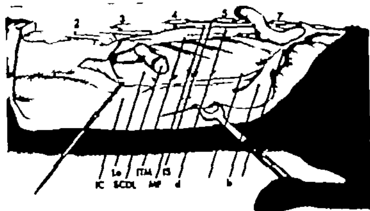
Results

Gross muscular morphology

To be able to analyze the action of the lumbar back muscles exact data on their morpho... and the associated connective tissue structures are of primary importance. The ava... literature seems however to give only generalized descriptions of these muscles in carni... and only few data have been found dealing specifically with the muscles in the cat. Va... discrepancies were also observed between different descriptions. Hence, a systemati... vestigation was undertaken to gain more knowledge of the morphology of the musc... the cat's lumbar back region.

The topographical relations of the muscles under study appear from Fig. 1-4. In ... is shown a dorsolateral view of the lumbar region and in Fig. 2 a cross section at the ... of the 6th lumbar vertebra. Laterally lie the iliocostalis (IC) and longissimus (Lo) mu... On the dorsal aspect of the longissimus the sacrocaudalis dorsalis lateralis (SCDL) a... intertransversarii mediales muscles (ITM) are located. The medial muscle group co... of the multifidi (MF) and interspinales (IS). The iliocostalis, longissimus, intertransv... mediales and multifidi muscles are arranged in parallel columns along the lumbar p... the spine while the interspinales are confined to the spaces between the spinous pro... (not illustrated in Fig. 2). All these muscles are found in more cranial regions of the tr... well. The sacrocaudalis dorsalis lateralis extends along the main part of the lumbar ... and ends at the level of the 2nd lumbar vertebra. Since all the muscles are located dor... lateral to the vertebral column the muscles execute other movements than flexion ... spine.

It is evident that the various groups composing the epaxial muscle system in the l... region show great variations in size. The longissimus and iliocostalis constitute alm...



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the total epaxial muscle mass in the region while the multifidi represent around 10 %, sacrocaudalis dorsalis lateralis about 8 and the intertransversarii mediales together with the interspinales only a few percent.

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Two separate fascial layers, attached to the iliac crest, cover the dorsolateral aspect of the back muscles (cut and retracted in Fig. 1). Dorsally they are attached to the spinous processes and the supraspinous ligaments. The thin superficial sheet covers all the muscles and inserts into the transverse processes and the intertransverse ligaments (*b* in Fig. 1). The inner layer, which is more prominent, covers the medial muscles and the dorsolateral surface of the longissimus and then enters the lateral muscle mass (in Fig. 1, 2).

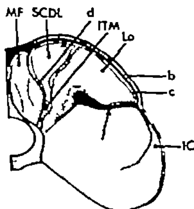


Fig. 2. Cross section at level of 6th lumbar vertebra. Fasciae and muscles marked as in Fig. 1. Darker dotted area of Lo shows localization of muscle tissue appearing red in color. Arrow indicates approximate position of fusion between lateral and medial connective tissue components of intermuscular septum.

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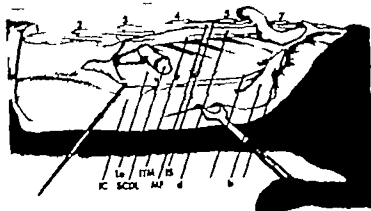
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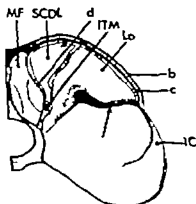


Fig. 2. Cross section at level of 6th lumbar vertebra. Fasciae and muscles marked as in Fig. 1. Darker dotted area of Lo shows localization of muscle tissue appearing red in color. Arrow indicates approximate position of fusion between lateral and medial connective tissue components of intermuscular septum.

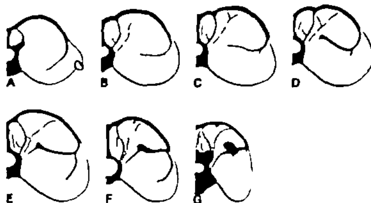


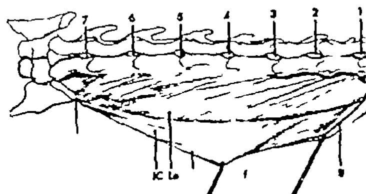
Fig. 3. Cross sections at [illegible] levels of lumbar region [illegible] organization of fasciae and [illegible] as well as outlines of muscles [illegible] Fig. 1 2). *A–F* sections at [illegible] of spinous processes of [illegible] lumbar vertebrae and G at [illegible] articulations between 6th and [illegible] vertebrae.

The fibers composing the inner layer run craniolaterad at the dorsolateral aspect [illegible] turn medially into the muscle mass. Within the muscle tissue there is a fusion between the fibers and others which run in the longitudinal direction. The arrow in Fig. 2 marks the approximate position of this fusion Thus a strong septum is formed which divides the lateral muscle mass into a ventral and a dorsal part. The septum runs along the back region in an almost horizontal plane and is attached to the iliac crest. This is borne out by the schematic cross sections at various levels of the lumbar region in Fig. 3 *A–G* At the levels above the 4th lumbar vertebra (*A–C*) the septum is thin and exclusively made up of fibers entering the muscle from the lateral side At and below the level of the 4th vertebra the septum is very prominent and located more dorsally than at higher levels (*D–G*). At the lower levels there is an additional thinner layer of connective tissue medial to the septum. At the most caudal levels this layer reaches the vertebrae (*F G*). The ventral part of the muscle mass is subdivided by a septum running in a nearly sagittal plane. This septum is well developed at low levels in the lumbar region (*D–F*) and appears to be a division of the inner prominent layer covering the dorsal aspect of the muscular system

Another fascia runs along the lumbar region and separates the medial muscles from the other ones (Fig. 1 *d* 2 *d* 3) The fascia is made up of fibers extending in dense bands from the mamillary processes towards the supraspinous ligaments and the spinous processes. Close to the midline the fascia adheres to the covering connective tissue layer (Fig 2). Within the compartment thus formed there are additional connective tissue structures but their organization has not been studied in detail.

Accordingly the back muscles in the lumbar region are divided into two compartments, *i.e.* a medial one which contains the multifidi and interspinales and a lateral one containing the iliocostalis, longissimus, intertransversarii mediales and sacrocaudalis dorsalis lateralis. The latter compartment is further subdivided by various septa composing surfaces on which the muscles insert. Hence they have an important function in the mechanics of the muscular system. Their design exhibited minor variations in the cats investigated

Previous descriptions of the lateral muscles in the lumbar region are somewhat conflicting. The longissimus, as described by Reighard and Jennings (1961) and by Taylor and Weber (1951), fills out the major part of the space between the spinous and the [illegible] processes. According to the same authors the iliocostalis is a th[illegible] lo[illegible] to the longissimus and distinguishable in the uppermost lumbar [illegible]



4 Dorsal ... of lumbar region. Structures marked as in Fig. 1. Muscles removed and inner fascial layer ... to show longissimus and its tendinous origins ( ) at accessory processes (f). I ... upper region ... bundles (g) insert into inner dorsolateral surface of fascia. Iliocostalis lies ventral to longis... and appears indistinctly through fascia.

...criptions the iliocostalis is connected to the ilium and the well developed connective ...ue septum is regarded as a border between the two muscles in the lumbar region of cats ...well as dogs (Bogorodsky 1930, Harreveld and Kok 193.., Rijnberk and Kaiser 1935). ...so in the present study the septum is considered as a border between the longissimus and ...costalis but it should be emphasized that other interpretations may be justified as well.

The longissimus muscle extends from the lateral aspects of the vertebrae and the accessory ...ocesses. The muscle fibers run caudolaterad and insert onto the medial parts of the prom... ...ent intermuscular septum and the medial aspect of the iliac bone (Fig. 1 2). Superficial ...rtions of the muscle appear pale in contrast to deep and medial, / central, bundles ...hich are red in color. The position of the red portion of the muscle is indicated in Fig. 2 ...y a darker dotted area. The portion lies immediately dorsal to the intermuscular septum ...nd can be seen along that part of the lumbar region where the septum is well developed, ...e. from the level of the 4th lumbar vertebra to the iliac crest. At the lower levels of the region ...he red bundles are limited in the ...entral direction also by the thinner septum (cf. Fig. 2 ...and 3 *E, F G*). In other directions there are no sharp borders to the pale muscle tissue.

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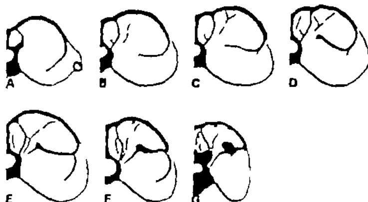


Fig. 3 Cross sections at sev levels of lumbar region show organization of fasciae and septa as well as outlines of muscles (cf. Fig. 1 2). *A–F* sections at le of spinous processes of 1st–4 lumbar vertebrae and *G* at level articulations between 6th and vertebrae.

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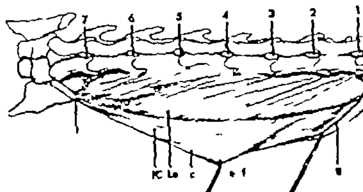


Fig. 4 Dorsal view of lumbar region. Structures marked as in Fig. 1. Muscles removed and inner fascial layer retracted to show longissimus and its tendinous origins (e) at accessory processes (f). 1 upper region ... bundles (g) insert into inner dorsolateral surface of fascia. Iliocostalis lies ventral to longissimus and appears indistinctly through fascia.

scriptions the iliocostalis is connected to the ilium and the well developed connective tissue septum is regarded as a border between the two muscles in the lumbar region of cats as well as dogs (Bogorodsky 1930, Harreveld and Kok 1932, Rijnberk and K...ber 1935). Also in the present study the septum is considered as a border between the longissimus and iliocostalis but it should be emphasized that other interpretations may be justified as well.

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Fig. 4 gives a dorsal view of the muscle. The fascial layers are retracted and the muscles located medially and dorsally have been removed in order to expose the oblique tendon-like formations on the dorsal surface of the longissimus. These structures originate from the accessory processes of the last thoracic and the upper six lumbar vertebrae and become progressively thinner as they extend caudolaterad. The areas between the tendons are covered by a thin fascia separating the underlying muscle from the medial intertransversarii and the tail muscle. The figure also demonstrates that muscle fibers originating at thoracic vertebrae insert onto the lateral and dorsal inner surface of the covering layer (in contrast to the insertions of the lumbar segments of the muscle). The longissimus is multisegmentally innervated by intermediate branches of the dorsal rami. The nerves enter the muscle at the various segmental levels just beneath its dorsal surface.

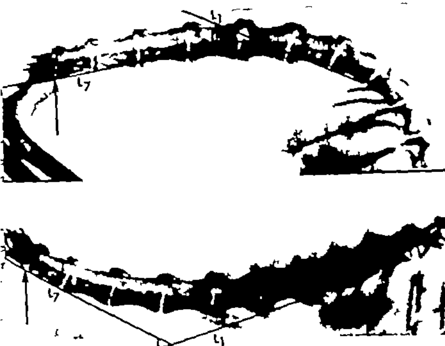
The iliocostalis muscle lies ventral and lateral to the longissimus. Caudally the muscle [illegible] connected to the iliac bone while the cranial portions insert onto the ribs. Superficial bundles are attached to the outer surface of the layer covering the longissimus muscle. The fibers extend cranioventrad to insert into the fascia which limits the muscle in the ventral direction (Fig 2) Deeper portions of the iliocostalis are connected to the ventral aspect of the [illegible] muscular septum as well as to the septum running sagittally at the lower levels in the (Fig 3 *E, F*) These portions extend towards the transverse processes, the intertra[illegible] ligaments and the lateral aspects of the vertebrae. The nerves supplying the muscle [illegible] lateral divisions of the dorsal rami and are located ventral to the intermediate [illegible]

The sacrocaudalis dorsalis lateralis and *the intertransversarii mediales muscles* are located at the medial dorsal aspect of the longissimus. The tail muscle lies most lateral and its fibers run dorsocaudad The muscle belly extends from the level of the 2nd lumbar vertebra towards the tail In the lumbar region this muscle inserts onto a narrow strip along dorsal surface of the longissimus (Fig. 1 2). In lower lumbar sections of the muscle there dorsally located tendon which runs caudad (Fig. 3 *F G*). In the sacral region it gives [illegible] pair of small tendons inserting onto sacral spinous processes. The nerve supply to the muscle differs from that to the other back muscles studied. Branches of the sacral [illegible] nerves collect to a common nerve trunk passing between the sacrocaudalis dorsalis lateralis and the longissimus muscles The nerve ascends into the lumbar region, taking a long [illegible] muscular course

The observations made in the present study show that the tail muscle, which some authors have described as the medial division of the longissimus (Reighard and Jennings 1961 [illegible] and Weber 1951) is actually a discrete muscle belly connected to the caudal skeleton Stimulation of the motor nerve results in tail movements. The muscle seems to be analogous to the sacrocaudalis dorsalis lateralis muscle in the dog and it should thus be justified to [illegible] the same designation in the case of the cat (*cf* Schumacher 1910).

Medial to the tall muscle lie the short bundles of the intertransversarii mediales muscle (Fig. 1 2) These fibers connect the mamillary and the accessory processes by insertions [illegible] the tendons at the dorsal aspect of the longissimus. The bundles also take origin from the fascia on the lateral aspect of the multifidi. At low levels in the region the bundles are less prominent and pass over fewer intervertebral joints than at the upper levels. Fibers originating from the mamillary process of the 6th lumbar vertebra insert onto tendons connected to the 5th and 4th vertebrae. Hence not more than one vertebra is bridged. Muscle portions located in the upper region sometimes span over three vertebrae thus, *e.g* fibers connected to the mamillary process of the 4th lumbar vertebra reach the tendon attached to the last thoracic vertebra. In the uppermost lumbar region the bundles insert also onto the dorsal aspect of the longissimus not covered by tendons. The medial intertransversarii bundles are innervated by medial branches of the dorsal rami entering the muscle from its deep aspect

The multifidi and *the interspinales muscles* are located side by side in the medial compartment of the region. The short and oblique multifidi bundles are interwoven with each other and form an almost homogeneous column along the lumbar spine. The muscle fibers are attached to the mamillary processes and extend craniomedially inserting onto the adjacent lumbar vertebrae by a complex of connective tissue structures.



5 X-ray pictures of lower spine and pelvic region in lateral view Flexed (*A*) and extended state (*B*) al lines represent longitudinal axes of sacral bone (S), seventh (L_7) and first lumbar ertebra (L_1) In-ated are angles between L_7 and L_1, and between S and L_7 Lumbosacral joint at arrow P pelvic bone. te the large mobility of the lumbosacral joint as compared ith that of lumbar intervertebral con-tion

Between the spinous processes the interspinales muscles extend cranioventrad In addition the insertions on the spinous processes the muscle bundles also attach to connective tissue uctures hich run sagittally in the compartment The interspinal muscles could be identi-d at the different levels in the lumbar region but in some cats they were weakly developed tween the tw last lumbar ertebrae. The multifidi and the interspinales muscles are in-rvated also by medial branches of the dorsal rami. The nerves pass lateral to the superior ticular processes and enter the muscle tissue from the entral aspect.

As far as the muscles in the medial compartment are concerned, the present observations ccord with previous descriptions given g by Kruger (1927) and by Reighard and Jen-ings (1961) (*cf* also Slyper 1946) Thus, it is not possible to distinguish single muscle undles or different layers of bundles in the lumbar region.

Mobility of the lower spine

n ew of the morphological observations made on the longissimus and iliocostalis muscles as ell as on the associated connective tissue structures it seemed relevant to study the mobility of the lumbar region, especially in the sagittal plane.

Fig 5 shows X-ray pictures of the lower spine in a lateral view when flexed (*A*) and ex tended (*B*). By measuring the angles between the longitudinal axes of different vertebrae in

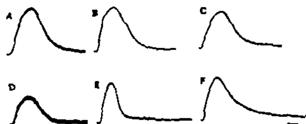
the flexed and in the extended position the mobility of different joints can be estimated the figure solid lines indicate the axes of the sacral bone (S), the seventh (L) and the lumbar vertebra (L). In flexion (*A*) the axes of the sacral bone and the seventh lumbar vertebra are practically parallel whereas the angle between the last and the first lumbar vertebra is about 30 In extension (*B*) the angle between the sacral bone and the last lumbar vertebra is 25 and between the last and first lumbar vertebra 45° The mobility of the lumbosacral joint (arrow) is thus 25 and between all lumbar vertebrae (L_7–L 6 levels) about Hence, the lumbosacral joint alone accounts for a large part of the overall mobility in lower spine and contributes significantly to the marked rotation of the pelvic bone (h the lower thoracic region the mobility of each joint is much more pronounced than in lumbar region

Contraction properties and observations on the action of some lumbar back muscles

The medial muscles multifidi and interspinales and the lateral longissimus and iliocostalis are supplied by multiple motor nerves entering each of the muscles at different levels in the lumbar region In order to find out more about this innervation pattern an initial series of experiments was performed in which the muscles were activated by stimulation of ventral roots or of the corresponding dorsal rami of the spinal nerves at different segmental levels Stimulation of ventral roots from different spinal segments proved to evoke contractions in different regions along the muscle bellies. Upper lumbar segments supply upper lumbar regions of muscles while lower segments innervate more caudal regions. The particular region of a muscle supplied by a particular segment could most readily be discerned by stimulation at a high frequency causing a tetanic contraction of the muscle fibers The myotomes of these muscles thus seem to be arranged longitudinally with a relatively small overlap between the adjacent segments. Stimulation of the dorsal rami at corresponding levels along the lumbar spine gave the same pattern. The findings indicate that the motor innervation of the muscles is closely correlated with the organization of the myotomes and exclude the existence of extensive nerve plexuses formed by the dorsal rami.

In these experiments the number of intervertebral joints directly affected by contraction of muscles innervated by one segment was also determined Stimulating the medial branch of the dorsal rami, which supplies multifidi and interspinales muscles, yields tension in tendon structures which run more or less sagittally in the medial muscle compartment and insert onto spinous processes (*cf* Fig. 1 2, 3). Maximum tension is recorded in the tendons which insert on the spinous process corresponding to the segmental nerve stimulated and in the two adjacent spinous processes. Thus, the findings demonstrate that medial muscles when activated by a given segment may act over intervertebral joints at least at three different levels.

Activating the longissimus and iliocostalis by stimulation of ventral roots at different levels results in a tension transmitted to large sections of the lumbar spine and to the pelvic bone. To establish possible differences in action between these muscles, they were selectively activated by needle electrodes and the tension development at the iliac crest as well as along the deep fascial layer was recorded (*cf* Methods). Activation of the longissimus in its middle and lower lumbar sections proved to result in maximal tension at the iliac crest while the



6. Isometric twitch contractions of medial muscles multifidi and interspinales (*A–C*) in response to supramaximal stimulation of their motor nerves arising from L_1 (*A*), L_4 (*B*) and L_6 (*C*) segment and of lateral muscles longissimus and iliocostalis (*D–F*) evoked by supramaximal stimulation of ventral roots L_1 (*D*), L_4 (*E*) and L_6 (*F*). Contraction time in *A* 42 ms, *B* 36 ms, *C* 39 ms, *D* 35 ms, *E* 26 ms and in *F* 27. Time bar 20 ms.

...sion along the fascial layer was lower. When stimulating the upper lumbar section of the longissimus more tension develops along the fascial layer than at the iliac crest. Contractions of the iliocostalis yield forces that are transmitted along the inner fascial layer and to the pelvic bone. Hence, the middle and lower parts of the longissimus seem to primarily act on the pelvic girdle. Other portions of the muscle operate on the lumbar spine as does the iliocostalis which in addition is connected to the pelvic girdle.

In view of the observations presented in the previous sections and the organization of medial myotomes an investigation of the contraction properties at different levels along the lumbar region was performed.

In Fig. 6 *A–C* are shown isometric twitch contractions at optimal muscle length of the medial muscles multifidi and interspinales in response to supramaximal stimulation of their motor nerves arising from the 1st (*A*), 4th (*B*) and 6th (*C*) lumbar segment (*cf.* Methods). In these recordings the contraction times (from onset to peak) vary between 36 and 42 ms.

The recordings in Fig. 6 *D–F* were made under conditions comparable to those in *A–C* and show isometric twitches in the lateral muscles longissimus and iliocostalis evoked by stimulation of ventral roots originating from the 1st (*D*), 4th (*E*) and 6th (*F*) lumbar segment. In these recordings the contraction times vary between 26 and 35 ms. The twitch responses in *E* and *F* show a longer relaxation phase than that in *D*. This phenomenon will be considered below.

Thus, it is evident that different regions of the medial muscles have about equal contraction times, as have also different regions of the lateral ones. However, the former muscles generally contract somewhat more slowly. This is borne out by Table 1 summarizing ex-

TABLE 1. Contraction times of cat lumbar back muscles.

Muscles	Segment	Range of contraction times (ms)	Mean contraction times (ms)	Number of experiments
Multifidi, Interspinales	L_{1-6}	36–42	34	10
Longissimus, Iliocostalis	L_{1-6}	26–35	29	15

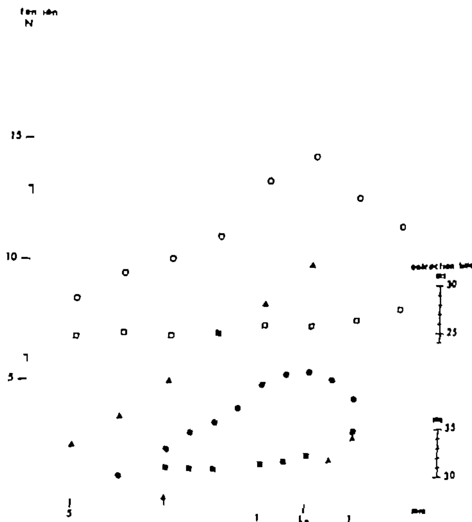
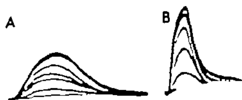


Fig. 7 Isometric twitch tension (circles), passive tension (triangles) and contraction time (rectangles) of medial muscles multifidi and interspinales (filled symbols) and of lateral muscles longissimus and iliocostalis (open symbols) plotted against muscle length. On abscissa L_0 marks muscle length giving biggest twitch amplitude.

perimental data on contraction times of the muscles (values obtained on stimulation of different segments have been pooled and mean values calculated).

As in the case of e.g. muscles in the hind limb the contraction times of the medial and the lateral muscles increase with lengthening of the muscles. The medial muscles exhibit the most prominent changes. This is illustrated in Fig. 7 in which the contraction times (rectangles) of the multifidi and interspinales (filled symbols) and of the longissimus and the costalis (open symbols) are plotted against muscle length. The twitch tension (circles) and the passive tension (triangles) developed in the muscles are plotted in a similar way. The maximal twitch tension in the lateral muscles is 2–3 times higher than in the medial ones. The former muscles are also capable to attain higher tension within a wider range of muscle lengths which indicates a difference in the length of the muscle bundles. Thus, e.g. at −4 mm the lateral muscles develop about 60% of their maximal tension but the medial muscles only about 20%. The passive tension recorded during lengthening of the muscles is



8 Superimposed recordings of isometric twitch contractions of medial muscles multifidi and inter-spinales (A) and of lateral muscles longissimus and iliocostalis (B) in response to stimulation of different strengths of the motor nerve arising from the L segment and the ventral root of the L segment respectively bars 20 ms.

h in both cases and this distinguishes them from the hind limb muscles where the tion is very low up to lengths resulting in maximal active tension (cf. e.g. Buller Eccles and Eccles 1960).

Muscle action potentials elicited by supramaximal nerve stimulation were of about the same duration in the medial as in the lateral muscles. The potential duration in the multifidi and interspinales ranged from 4.2 ms to 8.0 ms (mean 5.2 ms) and in the longissimus and iliocostalis from 4.3 ms to 5.0 ms (mean 4.7 ms).

Arrangement of motor unit populations

The contraction times of motor units are usually related to the diameter of their motor nerve fibers; thus fast units are innervated by coarser fibers than are slow units (see e.g. Wuerker, McPhedran and Henneman 1965). Since the threshold for electrical stimulation differs for nerve fibers of different sizes it is sometimes possible to distinguish between motor units with different contraction times by applying motor nerve stimulation of different intensities (cf. e.g. Andersen and Sears 1964).

Fig. 8 shows superimposed recordings from experiments utilizing this technique in which twitch contractions of the medial (A) and of the lateral muscles (B) have been elicited by stimulation of their nerves originating from the 4th and 5th lumbar segment respectively. The contractions elicited by low-strength stimulation in the former muscles attain peak tension somewhat more quickly than those elicited by activation of high-threshold fibers, thus indicating a mixture of motor units with different contraction times. The situation is essentially the same in the longissimus and iliocostalis muscles but in addition there is a late step in response to stimulation of medium and high-threshold fibers originating from the 4th lumbar segment (cf. also Fig. 6 E, F). This indicates the presence also of a slowly contracting population of muscle fibers in middle and lower regions of the lateral muscles which



Fig. 9. Reflex action potential (right) and isometric contraction (left) elicited in lateral muscles by electrical stimulation of the ipsilateral 4th lumbar dorsal root. Electrical response recorded from central region of longissimus at level of 5th–6th vertebrae. Time bars 2 ms (right), 20 ms (left).

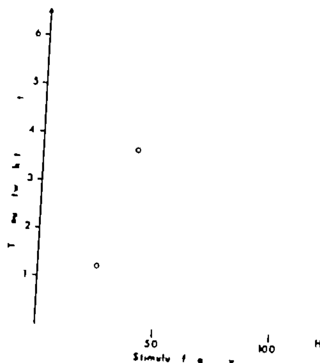


Fig. 10. Relation between stimulus fre-quency and tension developed in the medial muscles multifidi and interspi (triangles) and the lateral muscles longissimus and iliocostalis (circles)

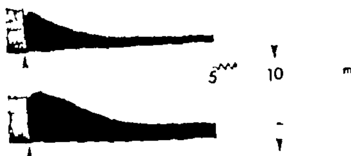
are supplied by lower lumbar segments. A heterogeneity of this kind is found in other musc of the cat as well (Denny Brown 1929 Gordon and Phillips 1953).

Further evidence for a slow component was obtained in experiments with reflex activa of the lateral muscles. Fig. 9 shows mechanical (left) and electrical (right) recordings fr the muscles in response to low-strength stimulation of the 4th lumbar dorsal root. The t to peak tension is about 100 ms and corresponds well to the late hump in the mechani response to ventral root stimulation (Fig. 8 *B*) The action potential is recorded from th central part of the longissimus muscle, located dorsally to the intermuscular septum compared with recordings from other parts of the muscles, the maximum amplitude of th electrical response was always recorded in this particular region of the longissimus.

The duration of the muscle action potential is 4.5 ms which is in the same range as the duration of the electrical response following ventral root stimulation. This implies that th time to peak tension cannot be attributed to repetitive discharges associated with the refle activation of the muscle but represents the contraction time of slow twitch units located i the longissimus. The nature of this reflex will be further dealt with in another paper (Carlso 1978 b)

Responses to repetitive stimulation

The mechanical responses to repetitive stimulation of muscle portions innervated from dif ferent segments were studied in a series of experiments, and in Fig. 10 typical results ar given. The figure shows the relation between stimulus frequency and tension developed and it appears that summation of mechanical responses in the medial muscles (triangle starts at 10–15 Hz and is total at about 50 Hz (apparent fusion *cf* Buller and Lewis 1965) whereas the corresponding values for the lateral muscles (circles) are 15 20 Hz and abo



11 T[illegible] contractions of multifidi and interspinales (*A*) and of longissimus and iliocostalis (*B*) [illegible] by supramaximal stimulation of their nerves from L_1 and L_4 segment respectively. Markings pointing [illegible] indicate stimulation at 5 Hz, downwards at 0.5 Hz.

[illegible] Hz. Summation of twitches started at a somewhat lower frequency in the middle and [illegible] regions of the two lateral muscles. This is likely to be due to the slowly contracting [illegible]ponent located in these regions.

Tetanus/twitch tension ratios were determined in 11 experiments on the two muscle [illegible] and proved generally to be higher for the lateral than for the medial muscles. The [illegible] for the former muscles ranged from 4.8 to 7.5 (mean 5.9) and for the medial muscles [illegible] 2.8 to 5.2 (mean 3.5).

[illegible]formance of lumbar back muscles during prolonged activity

[illegible] a total of 13 experiments the medial and lateral muscles were studied with respect to their [illegible] to maintain contractile tension during prolonged activity. Fig. 11 illustrates the [illegible]cline of twitch tension in the multifidi and interspinales (*A*) and in the longissimus and ilio[illegible] (*B*) during a period of motor nerve stimulation at 5 Hz. The recordings start when [illegible] twitches are obtained at 0.5 Hz. At the marking pointing upwards the stimulus fre[illegible] is instantly increased to 5 Hz. Following an initial staircase effect which is most [illegible] in the longissimus and iliocostalis, the twitch amplitudes decrease considerably [illegible] 10 min of stimulation [illegible] 5 Hz. At the end of this period the medial muscles retain [illegible] of their initial tension while the corresponding value for the lateral muscles is 21 [illegible]. The marking pointing downwards indicates a change of the stimulus frequency back to [illegible]5 Hz. T[illegible] contractions showing slight post-tetanic potentiation are then recorded in [illegible] muscle systems before a final twitch amplitude is reached which for the medial muscles [illegible] 24 [illegible] of the initial tension and for the lateral muscles 22 [illegible]. Recordings of compound [illegible] action potentials at different times after the onset of stimulation showed that the [illegible] from the multifidi and interspinales as well as from the longissimus and ilio[illegible] declined markedly during stimulation at 5 Hz. Thus, also other factors than a failure of the contractile mechanism seem to be responsible for the decrease of the contractile tension.

In most experiments involving muscle portions innervated by different segments, the medial muscles retained [illegible] higher proportion of the initial twitch tension than the lateral muscles during the later part of the period of stimulation at 5 Hz. During the subsequent

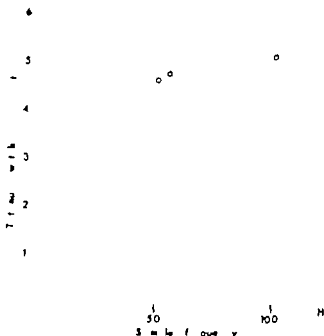


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and iliocostalis on the one hand and the pelvic girdle on the other hand is apparently major importance for the execution of movements and for the maintenance of stability hind quarters during different types of locomotion. In the uppermost lumbar region eptum is weakly developed and the mobility is greater Thus, it seems likely that the des in this region operate more exclusively on the axial skeleton than do the lower s of the muscles.

e study of the contractions in the longissimus also suggested a functional difference een the various sections of the muscle. Lower sections are probably more effective in control of the posture in the lumbosacral joint than are upper parts of the muscle. Since intervertebral joint is more mobile than other joints in the lumbar spine the longissimus ht to be capable of large ariations in length. Actually this is also the case, due to the ific morphology of the muscle. In middle and lower sections of the lumbar region the al aspect of the muscle is smooth, with no insertions onto the covering layer and may slide beneath the connective tissue sheet. In this context it is of relevance to state that slow component of the longissimus is able to extend the lumbosacral joint and might refore be expected to take part in the tonic control of the position of the pelvic girdle us the lumbar spine (cf Carlson 1978 b).

The medial division of the back muscles constitutes a minor portion and is formed by rt bundles connecting adjacent vertebrae. Contractions elicited by stimulation of a motor ve at given level affect only restricted sections of the lumbar spine. Hence, these bundles primarily involved in the control of movements and posture of one vertebra relative to other whereas the longissimus and iliocostalis act on larger sections of the lumbar spine. e medially located muscles do not exhibit any distinct separation into different layers. is might ell correspond to the limited mobility in the lumbar region since in other regions, ere the spine is more supple, the differentiation of the muscles into separate layers is more onounced.

nervation

he lumbar back muscles are supplied by the dorsal rami of the lumbar spinal nerves. The teral and intermediate branches of these rami innervate the iliocostalis and longissimus spectively and the medial branches the intertransversarii mediales, multifidi and inter pinales. The sacrocaudalis dorsalis lateralis is supplied by a separate nerve trunk, ascending rom the sacral region. In previous descriptions (Reighard and Jennings 1961 Taylor and Weber 1951) only two branches have been identified, viz. a medial and a lateral one. The resence of three separate branches of the dorsal rami has recently been observed also by Bogduk (1976).

In their investigations of the motor supply of the dog back muscles Harreveld and Kok 1932) and Rijnberk and Kaiser (1935) were able to demonstrate that specific segment supplies muscle fibers located in a particular region along the muscle bellies. The myotomes composing muscle were found to be arranged after one another in a row In the present experiments on the cat similar pattern was observed. Furthermore, stimulation of peripheral nerves entering the muscles at different levels along the spine was shown to evoke contractions in that part of the muscle which is activated by stimulation of the corresponding

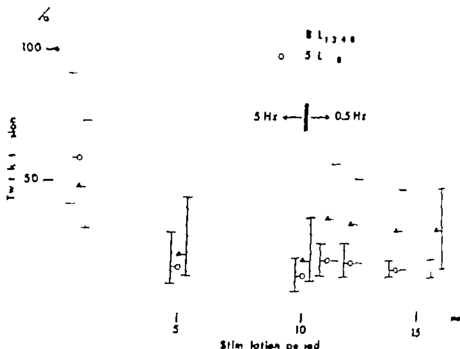


Fig. 1... Mean isometric twitch tension developed by multifidi and interspinales (triangles) and by lo aimus and iliocostalis (circles) on conti nous supramaximal nerve stimulation 5 Hz for 10 min follow 0.5 Hz for 6 min Bars indicate range of values. Segments investigated and number of experiments g figure.

period the difference between the muscles was more pronounced ($p < 0.001$ at 16 r Segmental variations in the susceptibility to fatigue may be present but this question not been studied specifically The experimental findings during prolonged muscular act have been summarized in Fig. 12.

Discussion

Gross muscular morphology

Great mobility of the axial skeleton is a characteristic feature of a cursorial mammal as the cat. The spine is flexed and extended during fast locomotion and hence such ani are called dorsomobile runners (Gambaryan 1974 cf Hildebrand 1959). During slo types of locomotion movements of the axial skeleton are less obvious (Goslow Rein and Stuart 1973) However the mobility in the vertical plane differs in various joints of axial skeleton as shown in the present study (Fig. 5). Each lumbar joint has less mob than the lumbosacral joint and the joints in the lower thoracic region which are bo mobile (cf Auer 1914 Slijper 1946).

The mobility of the axial skeleton is reflected in the morphology of the muscles and organization of the associated connective tissue structures. The lateral muscles longissi and iliocostalis are connected to the spinous processes by the inner fascial layer in the re (Fig. 1 2). This is essential for their action as extensors of the spine The two muscles also connected to the pelvic girdle by the intermuscular septum which is most prominen lower levels of the lumbar region The strong septum permits the force developed by muscles to be directly transmitted to the pelvic bone The associatio tween the l

...bar back muscles is fast contracting and shows a marked fatiguability on prolonged ...ulation and that only a small fraction of the muscle mass in the region may have tonic ...tions. This is of significance when considering their role in motor events.

... work was supported by ... grant from the funds of Karolinska Institutet.

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ventral roots. As indicated in Results, this implies that the multiple innervation is cor' to the organization of the myotomes. However the existence of nerve plexuses cann ruled out and this possibility should be further investigated.

This particular innervation pattern is similar to that of axial muscles in fishes and di' from the innervation of extremity muscles in mammals. In fishes myosepta separa myotomes from each other Such structures have not been found in the cat s dorsal mus Hence, the segmental organization of the myotomes should not be overemphasized Ne theless it is likely that different sections along a muscle are reflexly operated by differ spinal segments. Local differences in the activity of one and the same back muscle h been observed on application of nociceptive stimulation to back skin (Carlson and Ly quist 1976) Such differences can also be observed in the dog s back muscles during loc tion (Tokuriki 1973 1974) Observations of this kind indicate that the multiple innerv of the back muscles has direct consequences for the design and interpretation of experi dealing with reflex patterns as well as for studies of the activity of the muscles during lo motion.

Mechanical properties of lumbar back muscles

Since the total mobility of the spine is equal to the sum of the mobility of all the join might be expected that muscle bundles connecting adjacent vertebrae such as the multi and interspinales, should be able to develop high tension only within a narrow rang length in contrast to muscles controlling movements and posture over larger sections of t spine. The experimental results lend support to this hypothesis since the muscle leng could be shown to be more decisive for the twitch amplitude of the medial muscles than the longissimus and iliocostalis. This is probably due to differences in the organization of t whole muscles and should not be ascribed to differences in the properties of the individ muscle fibers.

The contraction times recorded for the lumbar back muscles are within about the sa range as those observed for some of the fast extremity muscles like gastrocnemius, fle hallucis longus and flexor digitorum longus (Wills 1942, Buller Eccles and Eccles 196 However the lateral muscles longissimus and iliocostalis contract slightly faster and are, general more susceptible to fatigue than the medial multifidi and interspinales. Furthermo the time course of the twitches in the latter muscles did not vary much in different regi along the lumbar spine, in contrast to the mechanical responses of the lateral muscles long simus and iliocostalis. Activating the latter muscles by ventral root stimulation of lo lumbar segments (L_4–L_6) suggested the presence of a slowly contracting portion. Re activation indicated that slow twitch fibers are located in the central region of the long simus muscle along the middle and lower lumbar spine. The differences in contract properties between the lateral and the medial muscles are in accordance with recent findi on the histochemical fiber composition of the various lumbar back muscles (Carlson 19 1978 a). Of course viscous and elastic elements may contribute to differences in contract properties between the various muscles as well as between different regions of a si muscle belly but these factors have not been analyzed specifically

Perhaps the most striking observations in the present study are that the main part of

...5.9 as is also the fusion frequency 70 Hz, and the fatiguability of these muscles is ...pronounced. Another difference is that a portion of the lateral muscles, located in the ...r region of the longissimus, contracts more slowly than upper parts. No such segmental ...ations were found in the medial muscles.

...e present work was undertaken to study the muscle fibers in the cat's lumbar back ...cles by histochemical methods in order to find out how the fiber composition of the ...rious muscles is related to their contraction properties. Thus biopsies from different ...ions along the muscles and at various depths were analyzed to determine the presence ... distribution of different types of muscle fibers. The analyses reveal that the muscles are ...posed of three main fiber types and that their distribution varies markedly in the ...erent muscles.

A preliminary account of some of this material has been given elsewhere (Carlson 1976).

Material and methods

...ochemical analyses. Biopsy material from different levels and regions of the longissimus, iliocostalis, ...ertransversarii mediales, multifidus, interspinales and the sacrocaudalis dorsalis lateralis (see Table I ...menclature as in Carlson 1978) was obtained from eight cats (3.2–4 kg) anesthetized by i.p. injec...n of Nembutal[a] (30–40 mg/kg b.w.). The biopsy material was immediately frozen in isopentane pre...ously cooled with liquid nitrogen. Sections 10–15 µm thick were cut in a cryostat at −20°C. Staining ...thods: myofibrillar adenosine triphosphatase (ATPase: Padykula and Herman 1955) following pre...cubation at pH 9.4, 4.6 and 4.3 (Brooke and Kaiser 1969, 1970), $NADH_2$ tetrazolium reductase ($NADH_2$-...R; Scarpelli, Hess and Pearse 1958), succinic dehydrogenase (SDH, Nachlas *et al.* 1957), phosphorylase ...achenda and Kanaki 1953 as modified by Eränkö and Palkama 1961), lipids (Carleton and Drury 1957) ...d glycogen (PAS technique as described by Dubowitz and Brooke 1973). Some slight modifications were ...ade (see Nyström 1968). For further details see Pearse (1960).

...eneral histochemical characteristics of muscle fibers

The histochemical characteristics of individual fibers were studied by photographic registration and com...parison of consecutive cross sections stained for the different enzymes and substrates.

Myofibrillar ATPase. In the sections preincubated at pH 9.4 two distinct fiber types can be identified in all the six muscles studied. The fibers that have a low activity of myofibrillar ATPase, type I in the nomen...clature by Brooke and Kaiser (1970), stain light and the fibers with a high activity, type II according to the same nomenclature, appear dark in the sections from the longissimus (Fig. 1 *A* and 2 *A*), the medial inter...transversarii (Fig. 3 *A* and 4 *A*), the multifidus (Fig. 5 *A*) and the interspinales (Fig. 6 *A*).

The staining pattern of the different fiber types may be altered by varying the conditions of preincubation (see e.g. Brooke and Kaiser 1970). Following preincubation at pH 4.3 the ATPase reaction is inhibited in type II fibers while no inhibition occurs in type I fibers. Hence, the staining pattern is reversed, type II fibers stain light and type I fibers dark. This reversal is evident when comparing the serial cross sections from the medial intertransversarii preincubated at pH 9.4 (Fig. 4 *A*) and at pH 4.3 (Fig. 4 *B*). However, certain type II fibers are not completely inhibited and stain moderately at pH 4.3. These conform to the type II C fibers in the classification by Brooke and Kaiser (1970) and in the figure such a fiber is indicated by a cross. In general, a very small number of type II C fibers were identified in the lumbar back muscles investigated and their relative proportions will therefore not be considered.

Preincubation at pH 4.6 results in a markedly inhibited ATPase reaction in some type II fibers but not in others. This modification of the ATPase reaction may therefore also be utilized to demonstrate further subdivisions among the type II fibers. This is illustrated in Fig. 1 and 5 showing consecutive cross sections from the longissimus and the multifidus muscles respectively. In Fig. 1 two type II fibers, indicated by an "a", stain dark for myofibrillar ATPase after preincubation at pH 9.4 (*A*) whereas only the lowermost fiber is inhibited and appears light in the section preincubated at pH 4.6 (*B*). Such fibers are termed type II A while fibers with intermediate stainability are designated type II B in the nomenclature applied

Acta physiol. scand. 1978. 103 198–209
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Histochemical fiber composition of lumbar back muscles in the cat

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Received 13 December 1977

Abstract

CARLSON H *Histochemical fiber composition of lumbar back muscles in the cat.* Acta physiol. scand. 1978 *103* 198–209

A histochemical analysis has been perf rmed of the activity of myof brillar ATPase, NADH-tetrazol. reductase, succinic dehydrogenase and phosphorylase and of the content of fat and glycogen in the m of the cat lumbar back region. The correlation between the f ber composition and the previously st contraction properties of the muscles was an lyzed. All muscles contain f bers with a low activity (ty and such with a high activity (type II) of myof brillar ATPase following preincubation at pH 9.4 Type fibers showed either a low (type II A) or an i termediate (type II B) reaction when stained for ATP preincubation t pH 4.6. Type I f bers have a high, II A an intermediate-high and II B f bers a low a of xidative enzymes. The longissimus, iliocostalis nd sacrocaudalis dorsalis lateralis muscles are cha terized by high percentages of type II B f bers and low proportions of type I and type II A fibers. T central region of the longissimus which is connected to a well developed intermuscular septum is c posed of high proportion of type I f bers. The multifid interspinales and intertransversarii med muscles ha e higher proportions of type I nd type II A fibers than the other muscles in the region.

The morphology and contraction properties of the lumbar back muscles in the cat w described in a previous paper (Carlson 1978) Medially located muscles such as the multifi and interspinales are made up of bundles taking a shorter and more oblique course than lateral longissimus and iliocostalis. Hence the medial muscles connect a smaller number vertebrae than the two lateral muscles which, in addition, are strongly connected to pelvic girdle. The back muscles have a multisegmental innervation the different spi segments supply different regions along a muscle belly in such a way that the myotom composing a muscle are arranged after one another in a row

The lumbar back muscles have short contraction times and fatigue fast on prolong stimulation. There are however some differences between the medial and lateral musc Thus the medial muscles as represented by the multifidi and interspinales have a mea contraction time of 34 ms, a tetanus/twitch ratio of 3.5 and an apparent fusion freque of 50 Hz. In the longissimus and iliocostalis which constitute the major portion of the later muscles the mean contraction time is somewhat shorter 29 ms, the tetanus/twitch ratio

I Biopsy material from lumbar back muscles.

	Vertebral levels	Number of specimens	Cat
...ostus			
...erficial	L_{4-5}	16	1–8
...p	L_4	3	3–5
...p and medial; "central"	L_{4-5}	7	5–8
...atus			
...erficial	L_{3-4}	12	1–8
...p	L_4		6, 8
...transversarii mediales			
...erficial	L_{4-5}	11	1 5 8
...ifidi			
...erficial	L_4	11	3–5, 7 8
...spinales	Th_{12}–L_4	7	4, 5 7 8
...quadratus lumborum laterales	not specified	4	2 5

1 C and 2 B represent sections stained for $NADH_2$-TR and demonstrate other dif... ...nces between the two parts of the muscle. I the central region (Fig. 2 B) the type I ...rs appear slightly lighter than they do in the superficial region. In the latter part of the ...scle (Fig. 1 C) the type II fibers exhibit varying staining intensities for the oxidative ...zyme while such gradations are less obvious in the type II fiber population of the central ...t of the muscle. Neither did specimens obtained from the latter region stained for ATPase, ...incubation pH 4 6, display any distinct differences between various type II fibers. Thus, a ...sification into subdivisions of type II fibers in the central part of the muscle becomes un... ...tain. In the peripheral part of the muscle, type II A fibers constitute about 14% and type ... B fibers 77% while the central portion is made up of about 31% of type II fibers. In the ...ter region type II C fibers are more numerous than in any other of the lumbar back ...scles investigated. When comparing the sections from the two regions of the muscle it ...also evident that the diameters of the fiber populations differ. In superficial portions type I fibers are small (38 μm) and the type II fibers considerably larger (mean value of

...able II. Histochemical staining reactions of lumbar back muscle fibers.

...staining	Type I	Type II A	Type II B
Myofibrillar ATPase preincubation at			
pH 9 4	low	high	high
pH 4 6	high	low	intermediate
pH 4 3	high	low	low
$NADH_2$-TR	high	intermediate–high	low
...DH	high	intermediate–high	low
...at	high	intermediate–high	low
Phosphorylase	low	high	high
Glycogen	low	high	high

Oxidative enzymes and fat. In Fig. 5 a type I fiber from the multifidi, lightly stained for myofi[illegible] ATPase preincubation at pH 9.4 (*A*) and darkly stained for ATPase preincubation at pH 4.6 (*B*), [illegible] dicated by an arrow also in serial cross sections stained for NADH$_2$-TR (*C*), SDH (*D*) and fat (*E*). [illegible] fiber is well supplied with the two oxidative enzymes and fat. Type I fibers in the lumbar back mu[illegible] exhibiting an intermediate stainability for the two oxidative enzymes and fat were only occasi[illegible] identified. In the same figure, two type II fibers darkly stained in *A* are indicated by an 'O'. The type II [illegible] fiber has an intermediate, and the type II B fiber reacting intermediate in *B*, a low activity of the oxida[illegible] enzymes and a low content of fat (*C*, *D*, *E*). Type II A fibers with a high activity for the oxidative enz[illegible] and a high content of fat are occasionally found in the muscles. Thus, in general type I fibers in the lu[illegible] back muscles have a higher stainability for the oxidative enzymes and fat than the type II A fibers [illegible] Fig. 1–5). Type II C fibers show a staining pattern for oxidative enzymes similar to that of the type I [illegible] fibers (Fig. 4). It is typical of the individual fiber that the reactions for NADH$_2$-TR, SDH and fat [illegible] parallel. This is in agreement with the staining patterns of extremity muscles in mammals. It is evid[illegible] that a separation of the type II fibers into two subgroups (corresponding to II A and II B) can be mad[illegible] the basis of the staining pattern in the reaction for NADH$_2$-TR as well as on the pattern observed in [illegible] tions stained for ATPase preincubation at pH 4.6. The former method was sometimes utilized to [illegible] such a separation since it was more often technically successful than the ATPase procedure.

Phosphorylase and glycogen. In Fig. 1 the staining reactions for myofibrillar ATPase, preincubation [illegible] pH 9.4 (*A*) and at pH 4.6 (*B*), NADH$_2$-TR (*C*), phosphorylase (*D*) and glycogen (*E*) are compared [illegible] serial cross sections from the longissimus. An arrow marks the position of a type I fiber which st[illegible] weakly for ATPase pH 9.4, phosphorylase and glycogen but gives an intense reaction for ATPase, pH 4.6 and NADH$_2$-TR. In the same figure two type II fibers are indicated by an 'O'. These fibers exh[illegible] high activity for myofibrillar ATPase, pH 9.4 and phosphorylase as well as a high content of glyco[illegible] while the stainability of the fibers for ATPase pH 4.6, and NADH$_2$-TR differs. Hence, the phosphoryla[illegible] activity and the glycogen content are largely parallel with the activity of myofibrillar ATPase after p[illegible] incubation at pH 9.4, i.e. low in type I and high in type II fibers.

The various types of fibers found in the six lumbar back muscles are summarized in Table II. The re[illegible] obtained show that the muscle fibers may be divided into three main types exhibiting staining patt[illegible] similar to those of fibers in hind limb muscles of the cat (Burke et al. 1973; for review see e.g. Close 197[illegible]. In general the type I fibers show a high stainability for the oxidative enzymes while the type II A fibers exhibit intermediate and the type II B fibers low reactions.

The relative proportions of different types of fibers in a specimen were calculated on examination of [illegible] bout 1 000 fibers except in specimens obtained from the interspinales and the lower parts of the [illegible] transversarii mediales muscles in which about 300 fibers were examined.

The size of the muscle fibers was determined in specimens from one cat by estimating the 'lesser fiber diameter' (Dubowitz and Brooke 1973) in cross sections stained for NADH$_2$-TR. Approximately 200–[illegible] fibers were measured in randomly selected areas of the cross sections following identification in consecutive sections stained for ATPase. Standard deviation of the mean fiber diameter was adjusted (Sheppard's correction, see Bailey 1969).

Results

Fiber composition of individual muscles

In total, 73 specimens obtained from various parts of the six muscles were examined (see Table I). The general histochemical characteristics of the muscle fibers are described above and summarized in Table II. The distribution as well as the diameters of the various fiber types in certain regions of the lumbar back muscles are summarized in Table III.

The longissimus. Fig. 1 *A* shows a cross section from a superficial muscle portion at the level of the 5th lumbar vertebra stained for myofibrillar ATPase, pH 9.4. Most fibers (91 %) are darkly stained and thus of type II. In Fig. 2 *A* is shown a central portion at the same level of the muscle stained for ATPase, pH 9.4. Macroscopically this part of the muscle appears red in color and is made up of a high proportion of type I fibers (69 %). These fibers stain somewhat more darkly for ATPase, pH 9.4 than the type I fibers in the peripheral region.



l. Serial cross sections through central region of longissimus at level of 5th lumbar ertebra. *A*, ..., pH 9.4 *B*, NADH$_2$-TR.

II A and II B 56 μm) whereas all the fibers in the central region are about equal in size e I 54 μm, type II 56 μm).

lopsies obtained from superficial as well as central regions also at other levels along the cle were analyzed with respect to fiber composition. Superficial portions at the levels m 7th to the 2nd lumbar vertebrae and specimens collected from the central region along ell developed intermuscular septum (*cf* Carlson 1978 Fig. 3 *D–G*) exhibit about the e fiber composition as that of the two regions at the level of the 5th lumbar vertebra. erficial bundles at the level of the first lumbar vertebra are composed of up to 20 % of e I fibers and about 14 % of type II A fibers. Hence, type II B fibers are less numerous in part of the muscle. Also specimens obtained from other deep regions than the central t of the muscle exhibited this composition of fiber types. In summary the peripheral ion of the longissimus muscle is mainly composed of type II fibers with a low oxidative ivity while the central part, connected to the most medial division of the prominent inter scular septum, has a high proportion of type I fibers.

The iliocostalis. In specimens obtained from superficial parts of the muscle at the level of e 5th lumbar ertebra type I fibers represent about 5 %, type II A 13 % and type II B 82 %.

... lumbar back muscles

...s med.		Intertransv. med. L_{4-5}			Multifidi L_{4-5}			Interspinales L_{4-5}		
II A	II B	I	II A	II B	I	II A	II B	I	II A	II B
22	13	12	23	65						
		17	26	57	26	24	50			
1	38	26	20	54	28	23	49	40	11	47
15	28	23	25	52	22	16	62	36	14	50
46	48	38	43	65	47	47	58	45	45	51
8.4	9.1	7.5	9.9	12.8	5.4	6.4	8.0	6.7	6.7	9.5

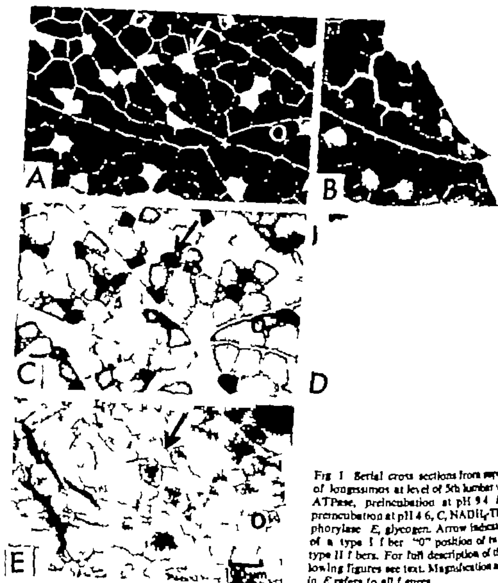


Fig 1 Serial cross sections from superficial of longissimus at level of 5th lumbar verte ATPase, preincubation at pH 9 4 B, ATPase preincubation at pH 4 6, C, NADH$_2$-TR, D phorylase E, glycogen. Arrow indicates ... of a type I fiber "0" position of two diff type II fibers. For full description of this and following figures see text. Magnification as indic in E refers to all figures.

TABLE III Frequency in percentages mean diameter (x) and standard deviation ( ) in μm of type

Cat no	Longissimus L_4 superficial			Longissimus L_4 central		Iliocostalis L_4 superficial			Sacrocaudalis dors. lat	
	I	II A	II B	I	II	I	II A	II B	I	II A
1	5	16	79			1	15	84		
2	8	12	80			4	7	89	8	13
3	6	13	81			4	13	83	7	14
4	12	17	71			8	14	78	19	17
5	11	12	77	66	34		13	80	18	[illegible]
6	11	13	76	72	28	9	14	77		
fiber diameter*										
$\bar{x}$	38	45	57	54	56	35	42	52	45	50
s	6.1	9.5	14.4	7.8	8.2	[illegible]9	8.3	10.7	7.1	7.8

* cf Methods, data obtained from cat no. 5.

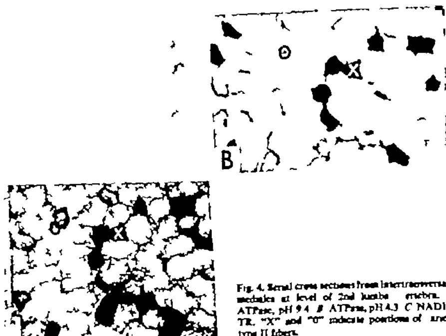


Fig. 4. Serial cross sections from intertransversarii mediales at level of 2nd lumbar vertebra. A, ATPase, pH 9.4. B ATPase, pH 4.3. C NADH₂-TR. "X" and "0" indicate positions of various type II fibers.

the type II fiber population are obvious. The type II fibers in the upper part of the muscle (Fig. 4) exhibit a broader spectrum of staining intensities which is exemplified by the three type II fibers indicated by an '0' in the figure. The lowermost of these three fibers shows weak reaction (type II B) while the upper two display different degrees of intermediate reactions (type II A). Fibers of the latter type constitute about 23% of the population at this level and thus type II B fibers amount to 57%. The diameters of the various type II fibers also differ markedly in this region (see Table III).

Biopsies also from other levels along the lumbar part of the muscle (L_4, L_4, L_4) were analyzed with respect to fiber composition. A gradual increase of the proportion of the type I fibers and corresponding decrease of the type II B fibers towards the lower levels of the muscle were apparent.

The multifidi. Serial cross sections at the level of the 5th lumbar vertebra are shown in Fig. 5. Type I fibers, lightly stained in *A* (ATPase, pH 9.4) and darkly in *B* (ATPase, pH 4.6), constitute about 25% of the fiber population. Type II fibers, darkly stained in *A* exhibit several gradations in the stainability for $NADH_2$-TR (*C*), SDH (*D*) and fat (*E*). The type II A fibers, reacting low in *B* and intermediate or high in *C*, *D* and *E*, form about 21% of the fibers. The type II B fibers, reacting intermediate in *B* and with a low stainability for the oxidative enzymes and fat, constitute about 54% of the fiber population. In the figure two type II fibers representative of the two subtypes are indicated by an '0'. Specimens collected from other levels along the lumbar region have a similar fiber composition. From the figure it is also evident that the type I and type II A fibers are small whereas type II B fibers are considerably larger (see Table III).

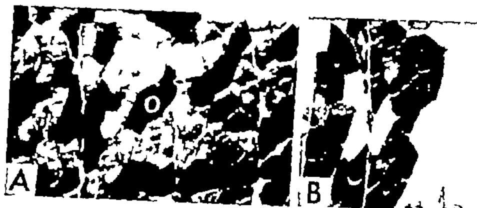


Fig. 3 Serial cross sections from *intertransversarii mediales* at level of 5th lumbar vertebra. *A*, ATPase pH 9.4 *B*, NADH TR. "O" indicates position of two different type II fibers.

Analysis of the fiber composition at other levels along the muscle (L_4–L) did not reveal great deviations from these values. However muscle bundles in the uppermost lumbar region inserting onto the ribs as well as deeper portions of the muscle are made up of about 20% of type I fibers, 15% of type II A and 65% of type II B fibers. Hence, the superficial and deeper regions of the iliocostalis have a fiber composition similar to that of the peripheral part of the longissimus. The diameters of the various fiber types in the superficial portions correspond to those of the fibers in the superficial longissimus muscle (cf Table II). In deep regions the type I fibers are somewhat larger than in superficial parts whereas the sizes of the various type II fibers are about the same in the two regions of the iliocostalis muscle.

The sacrocaudalis dorsalis lateralis In this muscle the type I fibers constitute about 13% type II A 17 and type II B 70%. However there are larger interindividual variations in fiber composition in this muscle than in the other lumbar back muscles (see Table III). The staining reactions as well as the sizes of the various fiber types correspond to those of the fiber population for example in the superficial parts of the longissimus muscle.

The intertransversarii mediales In Fig. 3 are shown sections of the muscle at the level of the 5th lumbar vertebra. Type I fibers lightly stained for myofibrillar ATPase, pH 9.4 in *A* constitute about 60% of the fibers. Various type II fibers are seen in the sections stained for $NADH_2$-TR *(B)* It is obvious that the fibers exhibit either an intermediate (type II A) or weak (type II B) reaction for the oxidative enzyme. Representative of the two subtypes are the fibers indicated by an 'O' in the figure. In this part of the muscle, types II A and II B constitute about 17% and 23% respectively of the total fiber count. The individual fibers are very similar in size and the diameters are estimated at 47 µm in type I, 46 µm in type II A and 48 µm in type II B

Fig. 4 illustrates another part of the same muscle, at the level of the 2nd lumbar vertebra. This part of the muscle differs in several respects from that in Fig. 3 Type I fibers appearing light in the section stained for myofibrillar ATPase after preincubation at pH 9.4 *(A)* and dark when stained for ATPase, pH 4.3 *(B)* constitute only about 20% of the fiber population The diameter of the type I fibers is also lesser than in the lower part of the muscle (see Table III). When comparing the sections stained for $NADH_2$-TR (Fi B 4 C) differences

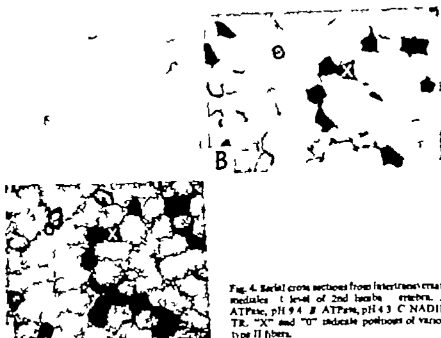


Fig. 4. Serial cross sections from intertransversarii mediales (level of 2nd lumba vertebra. A, ATPase, pH 9.4. B ATPase, pH 4.3. C NADH₂-TR. "X" and "0" indicate positions of various type II fibers.

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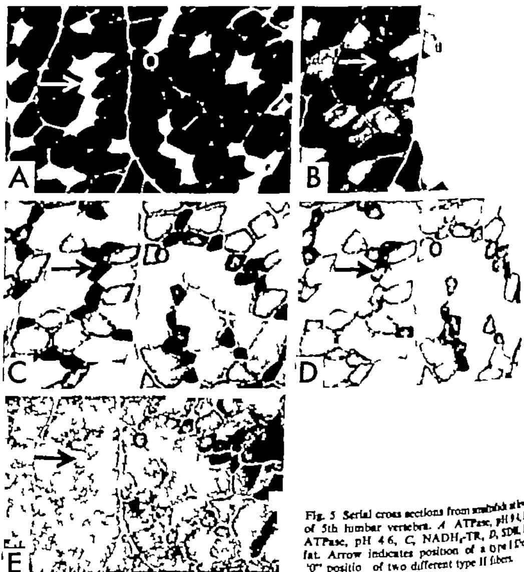


Fig. 5 Serial cross sections from multifidi at le[vel] of 5th lumbar vertebra. *A* ATPase, pH 9.4; *B* ATPase, pH 4.6, *C*, NADH$_2$-TR, *D*, SDH, *E* fat. Arrow indicates position of a type I fi[ber]; "O" positio[n] of two different type II fibers.

The interspinales These bundles lie just medial to the multifidi and have a different fi[ber] composition which is evident when comparing Fig. 5 (multifidi) and Fig. 6 (interspin[ales] L ₋₅). Type I fibers, lightly stained in Fig. 6 *A* (ATPase, pH 9.4) constitute about 38% of the fiber population. Type II fibers, appearing dark in *A* show low to high stainability in *B* (NADH$_2$-TR) Those reacting intermediate or high (type II A) make up about 14% and the pale fibers (type II B) 48% of the total population Specimens from other interspinal spaces were also analyzed and displayed only minor variations in fiber composition as specified above.

When comparing the fiber composition of different lumbar back muscles it is obvious that there are large differences in the proportions of type I and type II B fibers whereas the differences in the proportion of type II A fibers are less pronounced

Muscle spindles are found in all the muscles investigated An ... n of th



...I Serial cross sections through interspinales between spinous processes of 4th and 5th lumbar ...; A, ATPase, pH 9.4. B, NADH$_2$ TR.

...les in the various back muscles has not been made but a specific pattern of spindle ...bution in the longissimus muscle has been observed. In the peripheral part of the ...le, made up of a high proportion of type II fibers, the spindles are very sparse whereas ...are numerous in the central part which is mainly composed of type I fibers.

Discussion

...aim of this investigation was to characterize the lumbar back muscle fibers histo...ically and to relate the fiber composition of the various muscles to their contraction ...erties as described in a preceding paper (Carlson 1978).

...he staining pattern and the classification of the muscle fibers have been considered ...e. It has previously been shown that the biochemically estimated activity of myofibrillar ...Pase in smooth and striated muscles is higher the shorter the contraction time of the ...cle (Bárány 1967). With a histochemical mapping procedure (Edström and Kugelberg ...8) it has also been shown that fast-twitch units in the rat soleus muscle (Kugelberg 1971 ...3) as well as in the cat gastrocnemius muscle (Burke *et al.* 1971, 1973) are composed of ...ers with a high activity of myofibrillar ATPase (type II) and that slow-twitch units are ...de up of fibers with low stainability for the enzyme (type I).

The data obtained in the present study show that a very high proportion of the fibers in ...longissimus and in the iliocostalis muscles has a high activity of myofibrillar ATPase. ...proportion of such fibers is lower in the medial muscles multifidi and interspinales. In ...study of the contraction properties the lateral muscles longissimus and iliocostalis were ...t analyzed separately nor were the two medial muscles multifidi and interspinales. The ...stion then arises whether the contraction times and the fatigue properties observed are ...presentative of the individual muscles in view of their fiber composition. The multifidi ...scles form the main portion of the medial muscles while the interspinales constitute a very ...all part and hence the mean contraction time, 34 ms, is representative of the multifidi but ...t necessarily of the interspinales. The latter muscles may, in fact, be expected to contract ...ore slowly since their proportion of type II fibers is lower than in the multifidi.

Considering the small differences in the fiber composition of the two lateral muscles ...longissimus and iliocostalis it is apparent that the average time to peak tension, 29 ms, is

representative of both muscles. However the previous study also revealed a slow compa with a contraction time of about 100 ms located in the middle and lower parts of the lo simus muscle. This fits in with the findings in the present investigation. The central pa of the longissimus, located in close connection to the intermuscular septum, is made up high proportion of type I fibers. These are considerably larger than the type I fibers in periphery of the muscle and stain somewhat darker for myofibrillar ATPase, pH 9. addition, in stainings for oxidative enzymes the stained products are not as tightly as in other type I fibers. Histochemically these fibers are very similar to cat soleus (cf Nyström 1968 Burke *et al* 1974) It is possible that the type I fibers in the central pa of the longissimus represent a unique population relative to other type I fibers in the muscles. To provide further information on this point a comparative analysis of motor units is however necessary

In the central region of the longissimus, muscle spindles are more frequent than in o parts of the muscle. An association between spindles and muscle fibers presumed to be of slow twitch type is present in other muscles as well (see *e.g.* Richmond and Abrahams 19 The findings indicate that the proprioceptive reflex control differs in various parts of muscle.

In the longissimus and the iliocostalis, the predominant fiber type has a low stainab for oxidative enzymes and fat but stains more readily for phosphorylase and glyc (type II B). Fibers with this histochemical profile are apt to be depleted of their gly on prolonged stimulation and the motor units are found to be less resistant to fatigue th units composed of fibers with a higher activity of oxidative enzymes (Kugelberg and E ström 1968 Edström and Kugelberg 1968) On the other hand, in the multifidi and in t interspinales muscles about 50 of the fibers have an intermediate or high staining f oxidative enzymes. Thus, one would expect the medial muscles to be considerably m resistant to fatigue than the longissimus and the iliocostalis. However this is not the ca The difference in performance between the two groups of muscles is small the la muscles retain about 13% of their initial tension after a period of 10 min of low-frequ stimulation while the corresponding value for the medial muscles is 21 % (Carlson 197 In these experiments, signs of a failure in the impulse propagation were observed for b the lateral and the medial muscles. This indicates that the observed decline in contra tension may not exclusively be attributed to exhaustion of energy stores in the muscle fib

The results obtained in the previous investigation of the morphology and contract properties of the lumbar back muscles demonstrated a functional differentiation betw the various muscles. By the present analysis of their enzyme activity and substrate conc this differentiation has been further clarified.

This work was supported by gra t from the funds of K olinska I stitutet.

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...metric measurements. The following anthropometric measurements were performed: body ...and height, femur-condyle and radio-ulnar widths and skinfolds of subscapula, triceps brachii, ...brachii, and suprailiac area (Durnin and Rahaman 1967). For correlative analysis, fat-free body ...was also estimated according to von Döbeln (1959).

...olar power was determined by running maximally for a few seconds in a staircase as described by ...ria *et al.* (1966). For further details see Komi *et al.* 1977.

...metric forces were measured in maximal voluntary isometric extensions of the right knee (*quadriceps* ...) and of both legs (*total leg force*), respectively. *Force-time* curve was registered during the total leg ...measurements and the time to reach 70% of the maximum force was taken as force-time value (for ... Thorstensson *et al.* 1976).

...grated electromyographic activity (*IEMG*) was picked up from both m. rectus femoris and m. vastus ...is during maximum right knee extension (Viitasalo and Komi 1975).

...onaximetric measurements were obtained from the vastus lateralis muscle with the constant current ...of using the following stimulus durations: 0.1 ms, 1 ms and 30 ms. The 30 ms duration was considered ...rheobase stimulus and was therefore used as a basis for calculation of the chronaxie value.

...obic power. Individual maximum oxygen uptake (V_{O_2} max) was determined during treadmill running ...ding to Saltin and Åstrand (1967). The Douglas bag technique was used for collection of expired air ...CO_2 and O_2 contents were measured on a Scholander gas analyzer.

...rt rate (*HR*) was recorded during the V_{O_2} max test and the HR-value at the termination of the run ...taken as the peak heart rate.

...ak blood lactate concentration was determined in arterialized finger tip blood samples taken 3 to 5 ... after the maximal treadmill run. Reagents and instructions of Biochemica Boehringer GmbH were ...for the determination of the whole blood lactate concentration.

...eletal muscle fibre composition. Two muscle biopsy samples were taken from the vastus lateralis muscle ...described by Bergström (1962). One biopsy sample was used for classification of muscle fibres into fast ...h (FT) and slow twitch (ST) types (Gollnick *et al.* 1972) by staining for ATPase as instructed by ...hale and Hermann (1955).

...scle enzymes. The second biopsy sample was used for determination of enzyme activities. The enzymes ...ssayed were Mg^{2+} stimulated ATPase, creatine phosphokinase (CPK), and myokinase (MK) ac...ding to Thorstensson (1976), lactate dehydrogenase (LDH) according to Sjödin (1976), phosphorylase ...se), hexokinase (HK), and Ca stimulated ATPase (Lowry and Passonneau 1972). Distribution of the ...muscle specific LDH isozyme, LDH-1, was determined in the muscle biopsy specimens as described ...Karlsson *et al.* (1974).

...tistical analysis. Ordinary statistical procedures have been employed to calculate the means, standard ...ations (S.D.) and correlation coefficients (*r*).

Results

...ble I summarizes the comparison in the different variables between male and female ...bjects.

Anthropometric variables. The major differences in body size between females and males ...ere present in weight, height (for both variables larger values in the males), skinfold data ...higher in the females) and fat percent (higher in the females) (Table I).

Performance variables. V_{O_2} max was higher in the males both in l · min^{-1} ml · kg^{-1} ...min^{-1} (Table I). Peak blood lactate tended also to be higher in the males.

Of the different strength and neuromotoric (including IEMG) variables studied the results ...f the females were from 51.7–84.6% of that of the males. Especially pronounced was the ...difference in times to reach the 70% isometric force level, which was almost twice (198.9%) ...onger in females. In chronaximetric measurements no or only minor differences were ...present between the sexes (Table I).

Skeletal muscle characteristics. The males tended to have a higher %ST fibre distribution

Acta physiol. scand 1978. 103 210–218

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Skeletal muscle fibre types, enzyme activities and physical performance in young males and females

By

P V Komi and J Karlsson

Received 22 December 1978

Abstract

Komi P V and J Karlsson *Skeletal muscle fibre types, enzyme activities and phys performance in young males and females* Acta physiol. scand. 1978. *103* 210-218

Differences in skeletal muscle characteristics, metabolic profiles and functional performance be males nd females were investigated using young (15–24 yrs) male and female twins as subjects. The parison included such variables as anthropometry muscle strength mechanical power maximum o uptake electrical activation of muscle, muscle fibre composition (m. vastus lateralis), and activi several keletal muscle enzymes. The results disclosed the following primary differences between male females 1 the various functional tests the performance of females was from 61 1 to 84 6 % of that m distribution of slow twitch f bres in m. vastus lateralis of the females (49 1 ± 7 7 %) was lower (p < .05 that of the males (55 9 ± 11 9) activities of enzymes Ca stimulated ATPase, CPK, phosphoryla LDH were higher (p < 05–.01) in the males, whereas the distribution pattern of LDH-I isozy higher (p < .05) in the females. A pronounced difference between the two groups was a about l longer rise time of isometric force in females. It is concluded that the males as compared to the fe demonstrate higher aerobic and strength perf rmance capacity more efficient neuromotoric output d contraction, more slow twitch muscle fibres and more pronounced contractile and glycolytic poten the skeletal muscles.

With the exception of a recent preliminary report (Hedberg and Jansson 1976) the knowl is scarce concerning possible differences in muscle characteristics and metabolic pr between males and females. The present study is based on data collected in an examin of monozygous and dizygous twins of both sexes (Komi *et al* 1976 and Komi *et al* 1 in respect to histological histochemical and physical performance variables.

Methodology

Subjects for the study were btained through the Population Register of Finland. They ere all serolog tested according to routine methods (f r ref Komi *et al.* 1976). The final sample was composed male (9 MZ and 11 DZ) and 11 female (6 MZ and 5 DZ) twin pairs, i.e. pproximately a similar dis tion of MZ and DZ pairs among males and females. W th the exception of one male pair (11 years ages ranged from 15 to 24 years with the means of 17.8 nd 19 4, respectively f males and fema

II Summary of the correlation matrix for performance variables showing the significant ($p < .01$) correlation coefficients for females (left) and males (right).

	Vo_2 max l min	Vo_2 max ml kg^{-1} min^{-1}	LA	RV	MP	TLF	QF	FT	% ST	
Vo_2 max l min	■ ■				.83	.46	.69			Males (MZ+DZ)
$\dot{V}o_2$ max ml kg^{-1} min^{-1}	.66	■ ■ ■	.56	.57						
Blood lactate (LA)			■ ■							
Running velocity (RV)				■ ■ ■	.54					
Muscular power (MP)	.83			.54	■ ■	.67	.82			
Total leg force (TLF)	73					■ ■	.82			
Quadriceps force (QF)							■ ■		−.55	
Force-time (FT)	.56							■ ■		
ST fibres (% ST)									■ ■	

in the females (p .05) but also a large range of ariation as expressed by ±1 S.D which s 11.9 in the males as compared to 7 7 in the females.

Approximately similar muscle enzyme activities were obtained for males as compared females in respect to MK, HK, LDH in the forward reaction ($LDH_{pyr \to la}$), MG and $^{2+}$ stimulated ATPases, CPK, Plase, and LDH in the backward reaction ($LDH_{la \to pyr}$) re higher (15–67%) in the males, whereas the distribution pattern of LDH-I demonstrated higher percentage in the females (55%).

omparative analysis

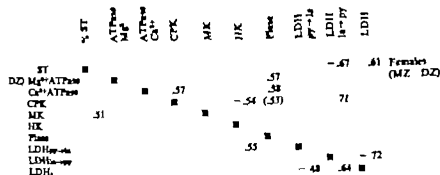
or the purpose of comparative analysis correlation matrices were formed to obtain inter elationships between all the variables studied. In the following only the most relevant information can be presented. For details, the reader is referred to Komi and Karlsson 1978. *Maximal oxygen uptake* ($\dot{V}_{O_2}$ max) expressed in l min^{-} was in the male material best orrelated to the following variables, muscular power (r = .83), quadriceps force (r = .69) nd total leg force (46) (Table 2). Similar correlations were obtained in the female material with the exception of quadriceps force. When V_{O_2} max was expressed per kg b.wt. in the males the best correlations were found to variables of peak blood lactate (r = .56) and running velocity (r = .57). In females skin variable was related to force-time (r = −.56) and in contrast to the males even to the alternate way to express aerobic power (l min^{-1} r = 66). *Muscular power* was in the two materials best related to V_{O_2} max expressed as l min^{-1} (r = .83 in both sexes, respectively) and running velocity (= .54 in both sexes). No significant

TABLE I Means ± S.D of the different variables for the monozygous (MZ) and dizygous (DZ) twins both sexes.

	Females MZ+DZ	Males MZ+DZ	Females/ males* (per cent)	t	p
1 Anthropometric variables					
Weight (kg)	50.8±7 7	62.6±12.7	81.2	3.99	[illegible]
Height (cm)	161 7±6.7	175.9±6.9	91.9	8.19	[illegible]
Femur-condyle lf	8.2±0.3	9.5±0.4	86.3	−12.17	[illegible]
widths (cm), rt	8.2±0.3	9 4±0.5	87.2	−11 60	[illegible]
Radio-ulnar lf	4 9±0.2	5.7±0.3	86 0	−9 83	[illegible]
widths (cm), rt	4.9±0.2	5 7±0.4	86.0	9 06	[illegible]
Scapula skinfold (mm)	9.9±3.5	8.6±4 4	115.1	1.20	[illegible]
Triceps skinfold (mm)	13.2±3.5	7.0±3.3	188 6	6 80	[illegible]
Biceps skinfold (mm)	5 1±[illegible]1	3 7±2.0	137.8	2 69	[illegible]
Crista ilica skinfold (mm)	6.7±3.4	7 6±5.0	88.2	0 84	[illegible]
Fat (%)	22.5±3.2	11.2±4.8	200.9	9 85	[illegible]
2. Performance variables					
V_{O_2} max (l × min⁻)	2.2±0.5	3 6±0.7	61 1	−8 40	
V_{O_2} max (ml × /kg min⁻¹)	43.2±4 7	56.6±5.7	76.3	−9 42	[illegible]
Peak heart rate	190.0±8.7	195.0±11.8	97 4	−1.74	[illegible]
Peak blood lactate	6.4±2.2	8.8±2.6	72.7	3 60	
Running velocity (m × s⁻¹)	1 1±0.1	1.3±0.1	84 6	8.73	[illegible]
Muscular power (kpm s⁻¹)	56.6±10.4	8[illegible]9±17 7	68.3	−6 53	[illegible]
Total leg force (kp)	171.9±30.0	214.2±54.5	80.3	−3.53	[illegible]
Quadriceps force (kp)	40.3±9 8	56.8±15.5	71.0	−4 67	[illegible]
Force-time (ms)	748.1±344.2	376.3±255 7	198.9	5.91	[illegible]
3 Muscle fibre composition					
ST fibres (%)	49 1±7 7	55.9±11.9	87.8	−2.49	
4 Muscle enzyme activities (moles × g⁻¹ min⁻¹ × activity constant)					
Mg^{2+} ATPase 10^{-4}	8.7 ±2.6	10.0 ±3.5	87.0	−1.20	
Ca^{2+} ATPase 10^{-4}	0.06±0.01	0.1 ±0.1	60 0	−2.11	
CPK 10^{-4}	0.02±0.01	0.03±0.01	66.7	−3.17	
MK 10^{-4}	80.6 ±23 7	74.2 ±23 6	108.6	0.34	
HK 10^{-4}	0.10±0.02	0.10±0.04	100.0	−0 48	
Pase 10^{-4}	0 70±0.4	1.05±0.6	66.7	−2.21	
$LDH_{py\to la}$ 10^{-4}	1.50±0.6	1 45±0.6	103.4	0.36	
$LDH_{la\to py}$ 10^{-4}	0.50±0.2	0.70±0.2	71 4	−2.18	
LDH 1 isozyme (%)	25.3 ±18.3	16.3 ±12.2	155.2	2.23	
5 Electromyographic variables					
IEMG, rectus femoris (mV × s⁻¹)	498 1±225.9	608.1±199.2	81.9	−1.63	
IEMG, vastus lateralis (mV s⁻¹)	281.4±165.5	544 6±408.3	51 7	−3.77	
6. Chronaximetric variables					
30 ms	4.0±1.0	3.9±1 4	102.6	−0.03	
1 ms	5.3±1.0	5.5±1.9	96.4	−0.14	
0.1 ms	16.5±5 1	19.9±13.8	82.9	0.78	

* Mean per cent value of the females as compared t the males.

III. Summary of the correlation matrix for muscle biopsy variables showing the significant ($p < 0.1$) correlation coefficients for males (left) and females (right).



	% ST	Mg++ ATPase	Ca++ ATPase	CPK	MK	HK	Phos	LDH py→la	LDH la→py	LDH1	
ST	■								−.67	.61	Females
DZ) Mg++ATPase		■					.57				(MZ DZ)
Ca++ATPase			■	.57			.58				
CPK				■		−.54	(.53)		71		
MK	.51				■						
HK						■					
Phos							■				
LDH py→la						.55		■			
LDH la→py									■	−.72	
LDH1								−.48	.64	■	

T as related significantly only to MK activity ($r = -.51$). This relatively high value sed the same relationship to be significant also in the total material (Fig. 2). In females T was related significantly to $LDH_{la \to py}$ and % LDH 1 (Table III, Fig. 3 and 4), respec ·ely with r .67 and r = 61

Discussion

The present study confirms the results of earlier studies that the males had a higher ysical performance capacity than the females. In respect to aerobic performance this was arly demonstrated and explained among others by Åstrand (1952). The difference in ength performance has not been as extensively investigated although the fact that the scle mass is larger in males has been pointed out as one contributory factor (Hermansen d von Dobeln 1971). In this connection it is important to emphasize the role of total mass determining the force production, because the force per unit of cross-sectional area of uscle is similar regardless of sex or to some extent of training (Ikai and Fukunaga 1968).

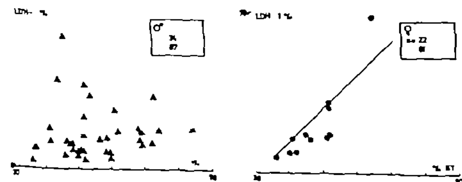


Fig. 3 Relationship between the distribution of isozyme LDH-1 and distribution of the slow twitch fibres of the vastus lateralis muscle in male (left) and female (right) subjects.

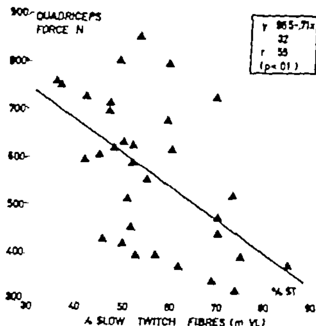


Fig. 1 Relationship between isometric force of the quadriceps muscle group (knee extension) and per cent distribution of slow twitch fibres (ST_{VL}) of the vastus lateralis muscle in all male subjects.

relationship was, however present between muscular power and percent distribution of ST fibres neither in males nor females. In addition, muscular power demonstrated in both sexes a high correlation to percent fat (r = 66 and 73 in the females and males, respectively).

Quadriceps force demonstrated in correlative analysis further differences between the sexes (Table II) Thus, in males total leg force (r = 82), muscular power (r .82), $\dot{V}_{O_2}$ max (l × min r = 69), percent ST fibres (r = −.55 Fig. 1) showed the highest correlations to quadriceps force.

Muscle fibre type distribution and enzyme activities Two major sex differences were observed when % ST fibres were correlated with the various muscle enzyme activities. In each

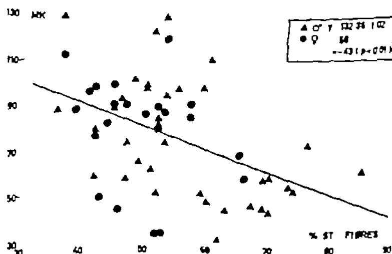


Fig. 2. Relationship between the activity of myokinase enzyme (MK) and per cent slow twitch fibres of the vastus lateralis muscle in all subjects including males and females. MK activity is expressed as moles × g^{-1} × min^{-1} 10^{-6}

y for lactate oxidation the per cent distribution of LDH-H isozyme was higher in the s. Normally quantitative and qualitative shifts in LDH isozyme patterns are ob- n relation to endurance training (Sjödin 1976). However it seems unreasonable to a training adaptation as an explanation for the observed sex differences. On the other the mechanisms operating during or initiated by training resulting in similar LDH ne shifts are also unknown.

qualitative differences between male and female skeletal muscle in terms of enzyme ties explains partly the observed differences in the force-time curves. It is likely that factors might also be involved in causing the difference in the force-time characteristics en males and females. It has been demonstrated that the elastic behavior of the es are different in males and females (Komi and Bosco 1977). It seems that the females more efficiently utilize the elastic energy which is stored in the activated muscle g prestretching. However they were relatively poor in jumping movement, which formed from a static starting position that allows practically no storage of elastic y. Again, however it is difficult to state whether these differences between the sexes xplainable by hormonal factors or training status. Irrespective the causal background, relationship between force-time and muscular power for both sexes (r = −.59 31) might indicate how the different variables are integrated in performance. The lower omotoric efficiency (represented by force-time) in combination with a qualitatively and titatively different skeletal muscle mass will result in a lower muscular power in the les.

summary the males demonstrated as compared to the females the following differences

- higher aerobic performance capacity
- higher strength performance capacity
- more efficient neuromotoric output during muscle contraction
- more ST muscle fibres as well as larger individual variations in their skeletal muscles
- more pronounced contractile and glycolytic profiles in their skeletal muscles

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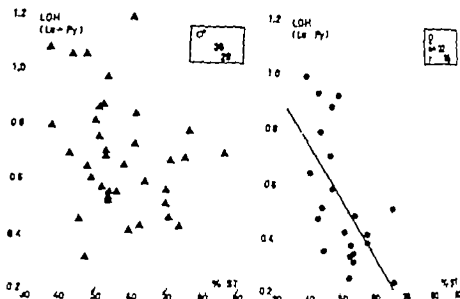


Fig. 4 Relationship between the activity of enzyme $LDH_{La \to Py}$ and distribution of the slow twitch fibres in the vastus lateralis muscle in male (left) and female (right) subjects.

If the total leg force in the present study is related to the body weight then the mean values are almost identical for the two groups 3.38 (females) and 3 40 (males).

The most important contribution from the present study is the differences between the sexes in terms of qualitative aspects on skeletal muscle. That the muscle fibre distribution is different has already been pointed out independently by a parallel investigation on males and females (Hedberg and Jansson 1976). The means were 53 ± 12.2 (S D.) and 49 ± 11 (S D) percent ST fibres, respectively *i.e* that not only the means are different between the sexes but the males have also slightly larger individual variation in muscle fibre composition. In addition to a higher percent ST fibres the males had also higher values for ATPase and CPK (Table I) These enzymes are normally more characteristic for the metabolic profile of the FT fibre (Gollnick *et al* 1974 and Thorstensson 1976)

The glycolytic profile seems also to be different and higher in the males. Preliminary studies with testosteron on castrated rats have demonstrated an increase in phosphorylase activity which is in line with the present data (Karlsson pers. com.). Further support for the concept of a more pronounced glycolytic profile in male skeletal muscle is given by the study of Hedberg and Jansson (1976), who demonstrated phosphofructokinase (PFK) activities corresponding to 15 69 ± 4 72 (S D) and 13 18 ± 3 06 (S D) in males and females, respectively The more pronounced glycolytic profile in males is also emphasized by the fact that the males have a larger percentage of ST fibres. Providing the samples have been obtained from a homogenous material the FT fibres normally possess higher contractile and glycolytic characteristics (Thorstensson 1976 and Sjödin 1976). The reason why the females differ in respect to qualitative aspects on skeletal muscle is unclear Although hormonal influences are indisputable as contributory factors, adaptation processes due to different physical activity patterns cannot be excluded.

In respect to the glycolytic profile it is of interest to note that in spite of the lower LDH

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Acta physiol. scand. 1978. 103. 219–224

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The release of serotonin from rat duodenal enterochromaffin cells by adrenoceptor agonists studied *in vitro*

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Received 31 January 1978

Abstract

PETTERSSON, G., A. DAHLSTRÖM, I. LARSSON, J. M. LUNDBERG, H. AHLMAN and J. KEWENTER. *The release of serotonin from rat duodenal enterochromaffin cells by adrenoceptor agonists studied in vitro.* Acta physiol. scand. 1978. *103.* 219–224.

The serotonin (5-HT) content of enterochromaffin cells (EC) was studied by a cytofluorometric method in specimens from rat duodenal mucosa after *in vitro* incubation with different adrenoceptor agonists and antagonists and acetylcholine (ACh). Noradrenaline (NA), Adrenaline (A) and Isoprenaline (IP) caused a decrease of 5-HT in EC down to 40–60 % and for NA and A this effect was concentration-dependent. The effect was antagonized by d,l-propranolol but not by d-propranolol, metoprolol, phentolamine or phenoxybenzamine indicating that the 5-HT release from EC is probably mediated via true β-adrenoceptor mechanisms possibly of the β_2 type. ACh also decreased the 5-HT content of EC but was much less potent than the adrenergic substances. Dopamine (DA) had no effect.

Key words: Rat enterochromaffin cells—duodenal mucosa—*in vitro*—serotonin release—cytofluorimetry—β-adrenoceptor

The enterochromaffin cells (EC) in mammals, such as mouse, rat, guinea pig, cat and man, have been shown to contain serotonin (5-HT) stored in large granules in the cytoplasm (Benditt and Wong 1957, Penttilä 1966). Populations of these cells have recently been demonstrated immunohistochemically to also store polypeptides, *e.g.* motilin and substance P (Pearse *et al.* 1974, Heitz *et al.* 1976, Polak *et al.* 1976). Thus, the EC of the gut fulfil the criteria of the so called APUD-group of endocrine cells, which can take up amine precursors, decarboxylate them and store the amine together with the hormonal peptide (*cf.* Pearse 1969).

The functional control of the EC in the gut is unknown but previous studies have suggested different possible mechanisms. 1) *Intraluminal stimuli*, such as instillation of hypertonic glucose or acidification of the duodenum cause degranulation of EC and a concomitant release of 5-HT to the blood (Drapanas *et al.* 1962, Fujita and Kobayashi 1971, Kellum and Jaffe 1976). 2) *Substances in the circulation e.g.* noradrenaline (NA) infusion to dog isolated

intestine, cause release of 5-HT into the perfusate (Burks and Long 1966) 3) Vernous stimuli may control release from the EC. For instance, stimulation of the cervical vagal nerve in cats causes a decrease of 5-HT in the EC of the small intestine (Ahlman *et al.* 1976 a). This was demonstrated by a direct cytofluorimetric technique where the 5-HT content in individual EC was estimated in samples taken before and after cervical nerve stimulation. This effect was mediated by vagal adrenergic fibres originating from the cervical sympathetic ganglia probably acting via a β-adrenoceptor mechanism (Ahlman *et al.* 1976 b, Ahlman *et al.* 1978 b Lundberg *et al.* 1978 a) Also, bundles of unmyelinated nerve processes including catecholaminergic (CA) fibres were observed electronmicroscopically near the basement membrane of all examined EC in the guinea pig duodenum (Lundberg *et al.* 1978 b) In the rat (Ohsumi *et al.* 1974) and the dog (Ahlman *et al.* 1978 a) a vagal adrenergic innervation from the cervical ganglia to the gastrointestinal tract has also been observed by fluorescence histochemical studies.

The aim of the present investigation was to study the receptor mechanism involved in the release of 5-HT from EC *in vitro*

Material and methods

18 Sprague-Dawley rat (males, 250 g) were killed by decapitation The proximal 5 cm of duodenum was dissected free and placed in icecold Krebs solution. The gut was opened to evert the mucosa, and then cut into 5 × 5 mm large pieces for further incubation.

The specimens were incubated for 30 min at 37°C in an oxygenated (5% CO_2, 95% O_2) Krebs solution containing the drug to be tested. In some experiments, where a receptor-blocking agent was to be tested, the specimen was preincubated with the blocker for 15 min before adding the agonist to the bath for further incubation (30 min). The controls in all experiments were incubated in Krebs solution for the same length of time

Drug

The drugs tested were noradrenaline (NA) (L-arterenol bitartrate Sigma Chemical Comp.), adrenaline (A) (L-epinephrine bitartrate Sigma Chemical Comp), isoprenaline (IP), (Isoprenaline sulphate Apotekbolaget), d pamine (DA) (3 hydroxy tyramine-HCl, Sigma Chemical Comp.) and acetylcholine (ACh) (acetylcholine-iodide BDH Chemical Limited) in the concentration range of 10^{-8} 10^{-3} M. ACh was always combined with eserine (Physostigmine sulphate BDH Chemical Limited) 10^{-4} M The blocking agent used were d l-propranol (Inderal ICI) 10^{-4} M d propranolol (Hässle) 10^{-} M metoprolol (Seloken® Hässle) 2 × 10^{-4} M phentol mine (Regitin® CIBA), 10^{-4} M phenoxybenzamine (PBA) (Dibenyline Smith, Kline and French) 10^{-4} M The bility of the blocking agents to antagonize the effect of A 10^{-} M was tested

Histochemistry and cytofluorimetry

After the incubation the gut specimens were immediately frozen in liquid propane freeze-dried and treated with para-formaldehyde vapour for fluorescence microscopy according to the Hillarp-Falck technique. The cytofluorimetric method has been described in detail earlier (Ahlman *et al.* 1976 a). From each specimen the 5-HT fluorescence intensity of 20 randomly chosen EC were recorded. As the relative measure of the 5-HT level of each EC the fluorescence intensity after 40 s of UV-illumination was recorded. (The reason for this is the rapid fading of the fluorescence see Ahlman 1976). These values were expressed in arbitrary units, and the mean value of intracellular 5-HT in each control sample (the specimens incubated in drug-free Krebs solution was set to 100 per cent The mean value and SEM for each incubated specimen were calculated and compared with the control from the same animal. In experiment where the effect of different receptor antagonist were tested the specimens incubated with both the receptor-blocker and the agonist were compared with the specimens incubated with the agonist only The results were statistically analysed by Student s t-test.

All specimens from one animal were processed simultaneously and all measurements were performed by the same person under identical conditions, using coded samples.

...e I. The effect of NA, A, IP DA and ACh incubation on 5-HT content of EC. Agonist concentrations expressed in M. Mean value and S.E. of 20 cells from each specimen. Difference from Krebs incubated control indicated by *(p < 0.01) and **(p < 0.005).

...ne	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	M
...±7.1	100±5.2		54±4.3*		35±2.4**	NA
...±7.1	96±6.1		75±5.6**		75±5.7*	
...±5.6	94±7.0		80±4.1**		68±5.1**	
...±3.3	76±3.9*	75±4.4	69±4.1	44±2.7**	35±3.2*	A
...±5.1	80±4.3*	80 4.3	63±3.2*	54±3.8*	46±2.5**	
...±4.0	81±3.9**	82+4.5*	57±3.8**	60+3.4	46±2.4	
...±4.3	73±3.4**		75±3.3**		83±4.3*	IP
...±6.3	57±3.7**		58±3.0**		38±4.5*	
...±5.9	75±3.4**		66±4.0**		68±3.5**	
...0±8.2			102±6.4		98±9.1	DA
...0±8.4	91±7.9		98±5.3		102±7.3	
...0+6.9	103±8.3		104±7.6			
...0±5.6	85_4.9*		83±7.1		88±7.3	ACh
...0+4.8	134+5.6		108±5.6		86±3.4**	
...0+4.4	93±4.4		84±4.0**		84±4.1	

Results

...he control specimens incubated in drug-free Krebs solution all showed a normal morphology ...ith well visible green fluorescent nerve terminals and strongly yellow fluorescent EC.

The effect of receptor agonists

...A clear-cut decrease of 5-HT fluorescence as compared to Krebs incubated controls was ...oted in the EC after incubation with NA, A and IP (Table I, Fig. 1). No difference be...ween the three amines was noted except that A and IP were effective in the lowest concentra...tion studied (10^{-9} M). The decrease in 5-HT fluorescence was concentration-dependent ...with NA and A (Fig. 1), while IP induced a maximal decrease already at 10^{-9} M.

DA did not affect the 5-HT content of EC in the doses tested (Table I). ACh in combina...tion with eserine caused a small release of 5-HT from EC but was clearly less potent than the adrenergic substances (Table I).



...Fig. 1. The relationship between NA-, A- and IP-concentration in the incubation medium and the release ...of 5-HT from the EC. Agonist concentrations expressed in M. For ... and S.E. see Table I.

intestine, cause release of 5-HT into the perfusate (Burks and Long 1966). 3) Nerves may control release from the EC. For instance, stimulation of the cervical vagal nerve in cats causes a decrease of 5-HT in the EC of the small intestine (Ahlman *et al.* 1976 a). This was demonstrated by a direct cytofluorimetric technique where the 5-HT content in individual EC was estimated in samples taken before and after cervical nerve stimulation. This effect was mediated by vagal adrenergic fibres originating from the cervical sympathetic ganglia probably acting via a β-adrenoceptor mechanism (Ahlman *et al.* 1976, Ahlman *et al* 1978 b Lundberg *et al* 1978 a). Also, bundles of unmyelinated nerve processes including catecholaminergic (CA) fibres were observed electronmicroscopically near the basement membrane of all examined EC in the guinea pig duodenum (Lundberg *et al.* 1978 b) In the rat (Ohsumi *et al* 1974) and the dog (Ahlman *et al* 1978 a) a vagal adrenergic innervation from the cervical ganglia to the gastrointestinal tract has also been observed by fluorescence histochemical studies.

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All specimens from one animal were processed simultaneously and all measurements were performed by the same person under identical conditions, using coded samples.

[illegible] mechanism (Nosal 1977). In all these instances the adrenergic release is probably one of several control mechanisms. The 5-HT release by adrenergic mechanisms may be [illegible]rt of the response caused by a general activation of the sympathetic system in a stress [illegible]tion. The ultrastructural finding of several types of nerve processes near the basement [illegible]brane of the EC indicate that the nervous control of these cells is not only an adrenergic [illegible] mechanism but far more complex (Lundberg *et al* 1978 b).

[illegible]rted by grants from the Swedish Medical Research Council (grants Nos. 17X 5220, 14X 2207 [illegible]170), by Göteborgs Läkaresällskap M Bergvalls Foundation, H. and G. Jeansson Foundation [illegible] by the Medical Faculty University of Göteborg. For generous supply of drugs we thank Hässle AB, [illegible], Sweden.

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TABLE II The effect of adrenergic blocking agents on A induced 5-HT release from EC. Agonist and antagonist concentrations expressed in M. Mean value and S.E. of 20 cells from each specimen. Difference from Krebs incubated controls ● $p < 0.005$. Difference from A (10^{-7} M) incubated specimens, $p < 0.01$ and $p < 0.005$

Krebs	A 10^{-7}	A 10^{-7} d,l prop 10^{-6}	A 10^{-7} d-prop 10^{-6}	A 10^{-7} Metopr 10^{-6}	A 10^{-7} Phentol-amin 10^{-6}	A 10^{-7} PBA 10^{-7}
100 ± 4.7	64 ± 3.5○	73 ± 3.9	55 ± 3.4	55 ± 4.2	59 ± 2.6	[illegible]
100 ± 4.3	67 ± 4.1●	100 ± 5.5	73 ± 4.2	60 ± 2.4	62 ± 6.0	$53 + 3$[illegible]
100 ± 5.0	57 ± 2.8●	69 ± 3.8	$55 \pm$[illegible]$.4$	40 ± 3.1	44 ± 2.0*	

2. *The effect of adrenergic blocking agents*

The ability of different adrenergic blocking agents to antagonize the release of 5-HT from EC caused by A (10^{-7} M) was tested (Table II). d,l propranolol a β-adrenoceptor blocking agent was the only substance which had a significant blocking effect. The other antagonists tested had no blocking effect on the 5-HT release in the concentration tested.

Discussion

The *in vitro* incubation technique used in this study has shown to be a useful tool for studying the 5-HT release from EC, caused by various receptor agonists. A careful handling of the biopsies during the incubation procedure was of great importance, but if this point was considered the effects of agonist incubation were very reproducible.

Earlier cytofluorimetric investigations indicated the presence of an adrenergic receptor on the EC in the cat small intestine, possibly on the β-type (Ahlman *et al.* 1976 b). The results of the present experiments on the *in vitro* effects of adrenergic agonists and antagonists on the rat duodenal EC confirm this observation. The effect was dose-dependent for NA and A. d,l propranolol, a β-adrenoceptor blocking agent had a blocking effect on the 5-HT release caused by A. This blocking effect was in all probability not due to a membrane stabilization, since d propranolol, having a membrane stabilizing effect only did not block the 5-HT release. Equipotent concentrations of metoprolol, a selective β_1-adrenoceptor blocker had no blocking effect. The results support the view that the adrenergic release of 5-HT from EC in rat was mediated via a true β-adrenoceptor mechanism, possibly of the β_2-type. Cholinergic receptors may also be involved, since ACh (10^{-6} M) had a small but significant effect on the 5-HT content of the EC (Table I).

It is possible, however that metoprolol, phentolamine, and PBA may have had an effect of their own in decreasing the 5-HT of the EC since the EC of specimens incubated with these blockers and A had a significantly lower 5-HT fluorescence intensity than incubated with A alone (Table II). A β-adrenergic release of hormones from endocrine cells has previously been suggested for the vagally induced release of gastrin in dog and man (Smith 1975 Christensen and Stadil 1976), for the adrenergic release of insulin from pancreatic islet cells (Lundquist 1971) and for the thyroxin secretion from the thyroid (Melander 1970). Another 5-HT-containing cell, the mast-cell has also been shown t have a β-ad

Acta physiol. scand. 1978. 103. 225–239

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Differentiation between pre- and postjunctional α-receptors in guinea pig ileum and rabbit aorta

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Received 4 July 1977

Abstract

[illegible], J. *Differentiation between pre- and postjunctional α-receptors in guinea pig ileum and rabbit aorta.* Acta physiol. scand. 1978. *103.* 225–239.

[illegible] stimulation of proximal and terminal guinea pig ileum induced contractions which [illegible] mediated [illegible] stimulation of cholinergic neurons. After blocking β-receptors, prejunctional α-receptors mediating [illegible] could be studied in these neurons. In proximal ileum the order of potencies of the following [illegible] was clonidine (8), epinephrine (3), oxymetazoline (2), norepinephrine (1), phenylephrine (0). The intrinsic activities of clonidine and phenylephrine were not maximal. In electrically stimulated [illegible] ileum practically identical results [illegible] obtained on the prejunctional α-receptor except that [phen]ylephrine was ineffective, the drug instead acted as [a] competitive blocker against norepinephrine. After blocking cholinergic receptors [wi]th atropine, excitatory [α]-receptors located postjunctionally to the [smo]oth muscle cells of terminal ileum could be studied. Under these conditions the order of potencies of [ag]onists was oxymetazoline (40), epinephrine (7), phenylephrine (4), norepinephrine (1), clonidine [illegible]2). In contracting postjunctional α-receptors in rabbit aorta the order of potencies [w]as oxymetazoline [illegible], norepinephrine (1), epinephrine (0.7), phenylephrine (0.2), clonidine (0.04). On the postjunctional [α]-receptor in terminal guinea pig ileum phentolamine was 55 fold more effective in blocking the response [o]f norepinephrine than [it] was in blocking the response on the prejunctional receptor of the same preparation. Phenoxybenzamine (4 · 10^{-8} M) [w]as ineffective on the prejunctional α-receptor of the cholinergic [neu]rons, [w]hile [it] blocked the postjunctional [α]-receptor of smooth muscle in guinea pig terminal ileum non-[illegible]ely. [It is] suggested that the differences in the activities of clonidine and phenylephrine between [pre]junctional α-receptors located to cholinergic neurons and postjunctional α-receptors located to smooth muscle cells was due to [a] pharmacological difference between the receptors. The results obtained [wi]th the [bl]ockers support this suggestion.

In an earlier paper (Wikberg 1977) results were presented which indicated that catecholamines induced relaxation of longitudinal smooth muscle of proximal guinea pig ileum by stimulation of α-receptors located to cholinergic neurons. The smooth muscle cells of this preparation lacked α-receptors although relaxatory β-receptors were present.

In two preliminary papers (Wikberg 1975, Wikberg *et al.* 1975) it was reported that the prejunctional α-receptor located to the cholinergic neurons of guinea pig small intestine showed a pharmacological difference in comparison to postjunctional α-receptors located to smooth muscle cells of guinea pig terminal ileum and rabbit aorta. Some of these findings

Relevant [illegible]

PEARSE, A. G. E., J. M. POLAK, S. R. BLOOM, C. ADAMS, J. R. DRYBURGH and J. C. BROWN, Enterochromaffin cells of the mammalian small intestine as the source of motilin. *Virchows Arch. Zellpathol.* [illegible] 111–120.

PENTTILÄ, A. Histochemical reaction of the enterochromaffin cells and the 5-hydroxytryptamine [illegible] of the mammalian duodenum. *Acta physiol. scand.* 1966. *Suppl.* 281

POLAK, J. M., P. HEITZ and A. G. E. PEARSE, Differential localization of substance P and motilin. *Scand. J. Gastroenterol.* 1976. *11* 39–42.

SMITH, C. L., J. KEWENTER, A. M. CONNELL, J. ARDILL, R. HAYES and K. BUCHANAN, Control [illegible] the release of gastrin by direct electrical stimulation of the vagus. *Amer. J. Dig. Dis.* 1975. 20 [illegible]

[illegible] of non linear least square regression analysis (Parker and Waud 1971). The function is [illegible] symmetrical, [illegible] curve [illegible] has plotted [illegible] semilogarithmic diagram. In the formula E is the effect, (A) the concentration of agonist, K the ED_{50} value, M the maximal effect and P a constant which is dependent on the [illegible] of the curve. Intrinsic activity ( ) has been defined

$$\text{i.a.} = \frac{M_{\text{tested substance}}}{M_{\text{norepinephrine}}}$$

[illegible] affinity

$$\text{rel.aff} = \frac{K_{\text{norepinephrine}}}{K_{\text{tested substance}}}$$

pD_2

$$pD_2 = -\log_{10} K$$

[illegible] ratios (DR) were estimated from the Ks of dose response curves in the absence and presence of antag-

$$DR_{i} = K_i/K_1$$

[illegible] K_1 [illegible] the ED_{50} of norepinephrine in the absence and K_i in the presence of blocker. The dose ratios [illegible] plotted by the method of Arunlakshana and Schild (1959). According to classical receptor theory [illegible] plot [illegible] $\log_{10}$ (B), (where (B) is the concentration of the blocker), to $\log_{10}$ (DR 1) should give [illegible] straight line [illegible] slope of unity. The intercept of this line [illegible] the $\log_{10}$ (B) axis gives the pA_2 for the blocking drug. [illegible] calculations the data [illegible] fitted into [illegible] straight line by [illegible] linear least square regression [illegible] the 95 [illegible] interval of the slope [illegible] estimated (Draper and Smith 1966). The pA_2 from the experiment [illegible] given by the intercept of [illegible] unity slope linear regression of $\log_{10}$ (B) to $\log_{10}$ (DR 1) to the $\log_{10}$ (B) [illegible]:

$$pA_2 \quad \left(\sum \log_{10} (B)_i \quad \sum \log_{10} (DR \quad 1)_i \right) /$$

[illegible] the apparent dissociation constant.

$$K_B \quad \exp_{10} (\quad pA_2)$$

[illegible] levels were calculated by analysis of variance and Students [illegible] test. Values in brackets after [illegible] determined values represent [illegible] 95 [illegible] confidence interval. Calculations were based on data [illegible] for [illegible] Hewlett Packard 9830 calculator and the results were processed graphically on [illegible] 9862 A plotter.

[illegible]

[illegible] following drugs were used: Atropine sulphate (ACO), cimetidine (SK&F labs), clonidine hydrochloride [illegible], dibenamine hydrochloride (Astra), dopamine hydrochloride (Sigma), l-epinephrine [illegible] (Sigma), haloperidol (Leo), histamine chloride (ACO), [illegible] hydrofumarat (Sandoz), [illegible] norepinephrine bitartrate (Sigma), oxymetazoline chloride (Draco), phenoxybenzamine (SK&F labs), [illegible] methansulphonate (Ciba), l-phenylephrine hydrochloride (Sigma), [illegible] chloride [illegible], [illegible] hydrochloride (Mead-Johnson), tetrodotoxin (Sigma), tolazoline hydrochloride (Ciba-[illegible]).

Results

[illegible] pig proximal ileum

Electric field stimulation of the guinea pig proximal ileum (coaxial stimulation) by 1 ms pulses at 0.1 Hz induced a twitch on each electric pulse. The twitches were completely blocked by tetrodotoxin (3 10 M) or atropine (3.5 10 M). A contraction elicited by histamine (7 10 M) was not at all affected by either tetrodotoxin or atropine (Fig. 1 A, B).

have recently been confirmed by Drew (1977 b) It has been suggested that a pharmacological difference exists between presynaptic α-receptors located to adrenergic nerves and postsynaptic α receptors located in smooth muscle of several adrenergically innervated organs (Starke *et al* 1974 1975 Langer 1974 Dubocovich and Langer 1974 Starke and Nelson 1975 Drew 1977 a)

The present study was undertaken with the intention to investigate whether a pharmacological difference between α-receptors located to cholinergic neurons and those located to smooth muscle cells was present or not α Receptors located to cholinergic neurons were studied in the electrically stimulated guinea pig proximal ileum (Paton 1955). α-Receptors located to smooth muscle cells were studied in rabbit aorta. In order to eliminate the possibility that a difference between the α-receptors was due to either species or organ differences, experiments were performed on the guinea pig terminal ileum as well. During electric stimulation α-receptors located to cholinergic neurons could be studied in this organ. After atropinization (Munroe 1951 1952) α-receptors located to smooth muscle cells could be studied in the same preparation.

Methods

M le guinea pigs or rabbits of either sex were killed by a blow on the head and bled. The smooth muscle organs were quickly removed dissected free from adjacent tissue and mounted in organ baths containing Krebs solution as described previously (Wikberg 1977). The Krebs solution always contained ... ($5 \cdot 10^{-6}$ M) ... ride to block β-receptors. The following preparations were used

Guinea pig proximal ileum. Coaxial stimulation of the guinea pig ileum was performed essentially as described by Paton (1955) A piece of the ileum was removed approximately 10 cm from the ileocaecal sphincter 2 cm long pieces were prepared and mounted on a silver wire which was also inserted into the lumen of the preparation serving as an electrode The silver wire was insulated when running outside the lumen of the intestine and was connected with a Grass FT 03 isometric transducer which permitted recordings of the longitudinal muscle activity on a Grass polygraf A second silver electrode was placed outside the lumen, in contact with the Krebs solution. At the lower end of the intestine the lumen was left open in order to avoid pressure differences between the outside and the inside of the preparation The preparation was given an initial load of $1 \cdot 10^{6}$ dyne and left for 1 h before the experiment was started. Stimulation was performed with a Grass SD9 stimulator and a home-constructed timer Supramaximal current with 1 ms rectangular pulses f frequency of 0.1 Hz was used. The luminal electrode served as anode

Guinea pig terminal ileum. 3 or 4 pieces of 1.5–2 cm length were removed from the terminal part of the ileum, starting where the small intestine joined colon. The preparation was mounted and stimulated electrically in the same manner as described for proximal guinea pig ileum above. The effects of agonists were first tested on the electrically stimulated preparation. Thereafter stimulation was stopped and the preparation was incubated with atropine $3.5 \cdot 10^{-7}$ M for at least 30 min. The agonists were then tested again on the same preparation.

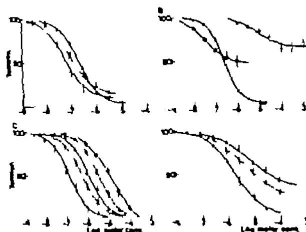
Rabbit thoracal aorta was removed and cut helically ... 4 ... 20 mm strips and mounted in the organ baths. The tension was recorded isometrically An initial tension of $2 \cdot 10^{6}$ dyne was applied to the preparation 1 h before the expt. was started.

In all expts when antagonists were tested, the preparations were incubated for at least 30 min with the drug before any expts. were performed.

Statistical methods

Dose response curves were treated statistically Each dose response curve was fitted into a logistic function

$$E = M\frac{[A]^{P}}{[A]^{P} + K^{P}}$$



3 Dose response curves by adrenergic agonists in the coaxially stimulated guinea pig proximal ileum. Continuous sigmoid lines represent the regressions of the logistic function to the experimental data. Epinephrine —, oxymetazoline ▲—▲, norepinephrine ●—● (n 7). B Clonidine O—O, norepinephrine ●—●, phenylephrine —, (n 8). C Test of tolazoline in the coaxially stimulated ileum. Norepinephrine was used as agonist. Control response by norepinephrine alone ●—●, tolazoline 10^{-6} M —, tolazoline 3 10^{-6} M ▲—, tolazoline 1 10^{-5} M O—O, tolazoline 3 10^{-5} O—O (n 4-5). D Test of phentolamine. Control response of norepinephrine alone ●—●, phentolamine 10^{-6} M —, phentolamine 2.7 10^{-6} M ▲ ▲ (n 6).

10^{-7} M), klemastin (3 10 M), thioridazine (4 10^{-} M) and haloperidol (2 10^{-6} M) almost completely inhibited the response to electric stimulation. Evaluation of these substances on eventual blocking abilities on the effect of norepinephrine in the electrically stimulated ileum was therefore impossible.

Table 1. Estimates of pD_2, relative affinity and intrinsic activity of adrenergic agonists in atropinized guinea pig terminal ileum, coaxially stimulated guinea pig terminal ileum and ileum and rabbit aorta

Terminal ileum	Atropine 3.5 10^{-7} M			Coaxial stimulation		
Drug	pD_2	rel. aff.		pD_2	rel. aff.	i.a.
Norepinephrine	34 3.79 (3.47–4.11)	1	1	6.67 (6.46–6.88)	1	1
Epinephrine	9 4.71 (3.98–5.43)	7	0.96 (0.82 1.10)	7.06 (6.48–7.65)	5	0.96 (0.92 1.01)
Phenylephrine	9 4.64 (4.36–4.91)	4	0.62 (0.37–0.86)	—	—	0 (see text)
Oxymetazoline	8 6.05 (5.66–6.45)	40	0.19 (0.08–0.30)	7.32 (7.00–7.63)	3	1.08 (0.99–1.16)
Clonidine	8 3.78 (3.45–4.11)	0.2	0.22 (0.12–0.33)	7.68 (7.38–7.98)	20	0.66 (0.41–0.91)
Dopamine	7 3.51 (3.28–3.74)	0.07	0.40 (0.23–0.57)	5.34 (5.22–5.46)	0.09	0.77 (0.64–0.91)

B	Rabbit aorta			Guinea pig ileum, coaxial stimulation		
Drug	pD_2	rel. aff.	a.	pD_2	rel. aff.	i.a.
l-Norepinephrine	15 6.47 (6.36–6.58)	1	1	15 6.53 (6.57–6.70)	1	1
l-Epinephrine	8 6.44 (6.24–6.64)	0.7	1.09 (0.74–1.43)	7 7.06 (6.58–7.54)	3	0.99 (0.95–1.04)
l-Phenylephrine	7 5.39 (5.31–6.07)	0.2	1.11 (0.55–1.67)	8 5.00 (4.68–5.33)	0.02	0.38 (0.22–0.54)
Oxymetazoline	7 7.05 (6.93–7.18)	3	1.12 (0.75–1.50)	7 6.86 (6.49–7.23)	2	0.95 (0.87 1.03)
Clonidine	8 5.19 (5.03–5.34)	0.04	0.13 (0.10–0.17)	8 7.51 (7.05–7.97)	8	0.52 (0.37–0.66)

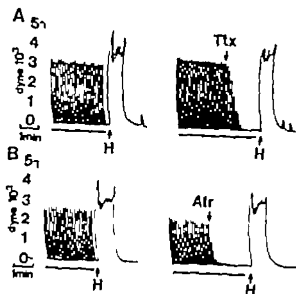


Fig 1 Responses of preparations from guinea pig proximal ileum. Coaxial stimulation 1 ms pulses at 0.1 Hz indicated by bars. A At arrows histamine 7×10^{-7} M and tetrodotoxin 3×10^{-7} M (Ttx). B At arrows histamine 7×10^{-7} M (H) and atropine 3.5×10^{-8} M (Atr). Washing indicated by dots.

In the experiments to be described sotalol (5×10^{-6} M) was always present in order to block β-receptors (Wikberg 1977)

Tests of agonists on the guinea pig proximal ileum. Typical responses of the drugs tested on the coaxially stimulated guinea pig proximal ileum are shown in Fig. Cumulative dose response curves of these drugs are shown in Fig. 3 A B and the results are summarized in Table I Both epinephrine and oxymetazoline were slightly more potent than norepinephrine The maximal inhibitory effect obtained by these drugs was not significantly different from that of norepinephrine. The intrinsic activity of phenylephrine was significantly lower (0.38 (0 22–0.54) p < 0 001) and its relative affinity only HC Clonidine also showed submaximal intrinsic activity in comparison with norepinephrine (0 52 (0.37–0 68) p < 0 001) Despite its low intrinsic activity clonidine was slightly more potent than norepinephrine.

Test of antagonists on the guinea pig proximal ileum The displacement of dose-response curves of norepinephrine by several drugs was investigated for the evaluation of their blocking properties in the coaxially stimulated guinea pig ileum. Several of the drugs that were tested were found to depress the electric twitch response markedly Thus, promethazine

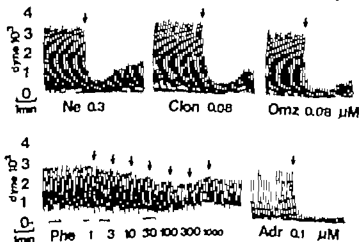
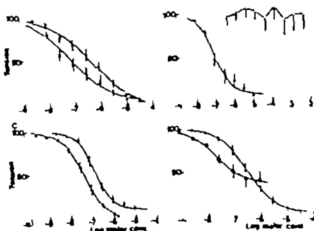


Fig. 2. Responses of preparations from guinea pig proximal ileum stimulated coaxially by 1 ms pulses at 0.1 Hz. Arrows indicate the addition of norepinephrine 3×10^{-7} M (Ne), clonidine 8×10^{-8} M (Clon) oxymetazoline 8×10^{-8} M (Omz), phenylephrine 1×10^{-6} – 1×10^{-3} M (Phe) and epinephrine 1×10^{-7} M (Adr).



4 Dose response curves of adrenergic agonists in the coaxially stimulated terminal ileum. A. Epinephrine — norepinephrine ●—● (9). B. Norepinephrine ●—● phenylephrine (n 5). C. Oxymetazoline ●—●, norepinephrine ●—● (5). D. Clonidine O—O epinephrine ●—● (5)

Test of cimetidine on the guinea pig proximal ileum. Experiments were performed to investigate if cimetidine (3 10⁻⁶ 3 10⁻⁵ M) influenced the response of oxymetazoline (10⁻⁶ M) and clonidine (1 10⁻⁶ M) in electrically stimulated guinea pig proximal ileum. Cimetidine had no blocking effect on the responses to the imidazolines (Table II A). There was possibly a significant potentiation of the effect of oxymetazoline by cimetidine (10⁻⁶ M) at p 0.05, however

Guinea pig terminal ileum

The terminal part of the guinea pig ileum responded to electric stimulation in a similar way as the proximal ileum. The electric stimulation was blocked by tetrodotoxin (3 10⁻⁷ M) or atropine (3.5 10⁻ M) while the response to histamine (7 10⁻ M) was not affected at all.

Test of agonists in the terminal ileum, inhibitory response. Norepinephrine, epinephrine, oxymetazoline, clonidine and dopamine depressed the twitch response to coaxial stimulation in the guinea pig terminal ileum in a similar way as was observed in guinea pig proximal ileum. Phenylephrine did not inhibit the contraction, however but instead often potentiated the twitch response to electric stimulation.

Cumulative dose response curves obtained on the coaxially stimulated terminal ileum, by these drugs are given in Fig. 4 and 6 A and the results are summarized in Table I A. Epinephrine and oxymetazoline were slightly more potent than norepinephrine. The intrinsic activity of epinephrine and oxymetazoline did not significantly differ from that of norepinephrine. Clonidine was about 20 times more potent than norepinephrine but its intrinsic activity was less (0.66 (0.41–0.91). The relative affinity of dopamine was 0.09 and its intrinsic activity was submaximal (0.77 (0.64–0.91)). Phenylephrine did not cause any dose related inhibition of the electrical stimulation. I three of the preparations there was an inhibition of the electric contraction to maximally about 75%. The rest of the preparations showed potentiation in all the concentrations tested (4) or slight inhibition in the lower and potentiation in the higher concentrations of phenylephrine. The variability of the responses did not allow any determination of the drug parameters.

TABLE II A. Effect of cimetidine on the inhibitory responses of oxymetazoline and clonidine on the ... stimulated guinea pig ileum. The effect of the agonists are given as the percental inhib... the twitch response. ±S.E.M. (n.s. = not significant)

Agonist	Cimetidine Control	3×10^{-6} M	1×10^{-5} M	3×10^{-5} M
Oxymetazoline (1×10^{-6} M) (n = 5)	56.9±2.9	67.1±4.4 $p < 0.05$	57.3±4.3 n.s.	57.0±5.5 n.s.
Clonidine (1×10^{-6} M) (n = 6)	46.7±5.3	53.5±6.4 n.s.	46.4±4.5 n.s.	35.9±4.4 n.s.

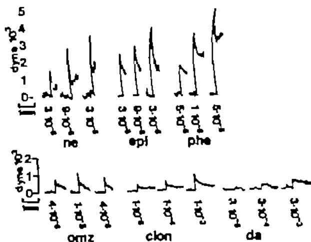
TABLE II B. Effect of tolazoline on the inhibitory responses of epinephrine, phenylephrine, oxymetazoline and clonidine on the coaxially stimulated ileum. The effect of the agonists are presented as in Table II A

Agonist	Tolazoline Control	1×10^{-4} M
Epinephrine (1×10^{-6} M) (n = 4)	91.1±0.8	16.3±9.3 $p < 0.01$
Phenylephrine (1×10^{-4} M) (n = 4)	32.8±3.4	18.9±3.2 $p < 0.01$
Oxymetazoline (1×10^{-6} M) (n = 4)	64.9±4.5	19.6±1.9 $p < 0.001$
Clonidine (1×10^{-6} M) (n = 4)	50.5±6.7	25.5±2.9 $p < 0.01$

Tolazoline (1×10^{-4}–3×10^{-4} M) caused a parallel displacement of the dose response curves of norepinephrine to the right (Fig. 3 C). Analysis of variance showed that there were neither any significant effects on the initial twitch contraction ($p > 0.25$), nor on the final maximal response caused by norepinephrine ($p > 0.25$) induced by tolazoline. Neither were there any effects on the slopes of the dose response curves ($p > 0.1$). The slope of the linear regression of log (B) to log (DR–1) (n = 19 from 5 expts.) was not significantly different from unity (0.96 (0.74–1.19)). The pA_2 calculated from the expts. was 6.4 (6.09–6.71) and the apparent K_b 4.0×10^{-7} M.

Phentolamine 8.0×10^{-7} M and 2.7×10^{-6} M depressed the twitch response by electrical stimulation to 75.3 (65.6–85.0)% and 52.2 (49.2–65.2)% respectively. The dose response curve for norepinephrine was not displaced in a typically parallel manner by phentolamine but the maximal inhibitory effect of norepinephrine was diminished (Fig. 3 D). The slope of the linear regression of log (B) to $\log_{10}$ (DR–1) was far different from unity (0.2 (−0.88–+1.33)) and there was no significant correlation at all in the regression ($p > 0.2$). The pA_2 calculated from the data was 5.96 (5.61–6.31).

The blocking properties of tolazoline on the responses of epinephrine, phenylephrine, oxymetazoline and clonidine were also investigated in the guinea pig proximal ileum. Tolazoline (1×10^{-4} M) counteracted the effect of epinephrine (1×10^{-6} M), phenylephrine (1×10^{-4} M), oxymetazoline (1×10^{-6} M) and clonidine (1×10^{-6} M) (Table II B).



7 Responses of adrenergic agents in atropinized guinea pig terminal ileum. Responses to norepinephrine 3 10⁻⁸ 3 10⁻⁶ M (ne), epinephrine 3 10⁻⁸ M (epi), phenylephrine 10⁻⁵ 10⁻⁴ M (phe), oxymetazoline 4 10⁻⁸–4 10⁻⁶ M), clonidine 1 10⁻⁸ 1 10⁻⁶ (clon) and dopamine 3 10⁻⁶ M (da)

the slope of the curve (p 0.1) or on the maximal response (p 0.8) (Fig. 5 B). The pA phentolamine was 5.39 (4.51–6.27) and K_B 4 1 10⁻⁶ M

Oxybenzamine (3 10⁻⁷ M) depressed the electric stimulation to 80 6 (72.4–88.8)%. The drug caused a displacement of the dose response curve of norepinephrine to the right without affecting the maximal inhibition induced by norepinephrine (Fig. 5 C). Phenoxybenzamine (4 10⁻⁶ M) slightly depressed the electric stimulation of terminal ileum. The electric contraction after phenoxybenzamine was 89 1 (65 1 113.0) of the control. The drug did not seem to have any blocking properties on the effect of norepinephrine in this concentration (Fig. 5 D). Since phenylephrine showed very little agonistic activity on the twitch contractions of the electrically stimulated terminal ileum it was of interest to investigate if the drug possessed blocking properties against norepinephrine in this preparation. Phenylephrine (5 10⁻⁴ M) caused a clear displacement of the dose response curve of norepinephrine to the right without affecting the slopes (p 0.5) or the maximal response (p 0.8). Neither did phenylephrine affect the contraction following electric

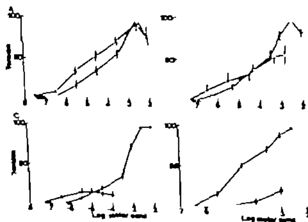


Fig. 8 Dose response curves of adrenergic agonists in atropinized guinea pig terminal ileum. A. Epinephrine — norepinephrine ●—● (n 5) B Norepinephrine ●—● phenylephrine — (n 5) C Oxymetazoline — norepinephrine ○—○ (5) D Norepinephrine ●—● clonidine ○—○ (8).

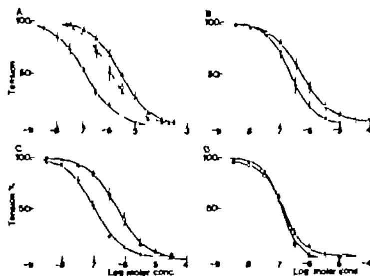


Fig. 5. Test of antagonists ... coaxially stimulated guinea pig terminal ileum. Norepinephrine was used as agonist in all experiments. Control response by norepinephrine alone ●—● A Tolazoline 3 10⁻⁶ M □—□, tolazoline 1 10⁻⁵ M ▲—▲ (n=6). B. Phentolamine 2.7 10⁻⁶ M □—□ (n 7) C Dibenamine 3 10⁻ M ○—○ (n 8) D Phenoxybenzamine 4 10⁻⁶ M ○—○ (n 6).

Test of antagonists in the terminal ileum, inhibition of inhibitory response Tolazoline (3 10^{-6} – 1 10 M) shifted the dose response curve of norepinephrine to the right (Fig. 5 A) Tolazoline did not significantly affect the initial twitch response by electr. stimulation ($p > 0.25$), the maximal inhibition induced by norepinephrine ($p > 0.25$) or the slope of the dose response curves ($p > 0.25$). The slope of the linear regression of the Schild plot was not significantly different from unity (0 73 (0 13 1.33)) (n = 12 from ... experiments) The pA of tolazoline was 6.38 (6.14–6 61) and the apparent K_B 4 2 10^{-7} M. Phentolamine (2 7 10^{-6} M) depressed the electric stimulation of the guinea pig terminal ileum to 79 0 (68 5–89.5) of the initial contraction The drug caused a slight shift of the dose response curve of norepinephrine to the right without causing any significant effect

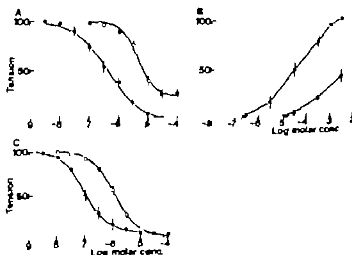


Fig. 6 A. Test of adrenergic agonists on coaxially stimulated guinea pig terminal ileum Norepinephrine ●—● dopamine ○—○ (=7) B. Tests on guinea pig terminal ileum pretreated with tropine 3.5 10^{-7} M ●—● dopamine ○—○ (=7). C. Demonstration of the blocking properties of phenylephrine on the response of norepinephrine in the coaxially stimulated guinea pig terminal ileum Control response by norepinephrine ●—● response of norepinephrine after 15 min pretreatment with phenylephrine 5 10^{-4} M □—□ (n = 4).

enoxybenzamine (4 10⁻⁸ M) depressed the maximal response to norepinephrine 9 C). Dibenamine (3 10⁻⁸ – 3 10⁻⁸ M) caused a similar depression of the maximal t obtained by norepinephrine as well as phenoxybenzamine (Fig. 9 D).

…bit aorta

…of agonists in the rabbit aorta. The -receptor agonists tested in the guinea pig ileum …r terminal ileum were for comparative purposes tested in the rabbit aorta. The results are …marized in Table I B. The maximal effects obtained by norepinephrine, epinephrine, …enylephrine and oxymetazoline did not differ significantly. Oxymetazoline was slightly …re potent while epinephrine and phenylephrine were slightly less potent than norepine…ine. The effect of oxymetazoline was slow at the onset and extremely persistant and did … disappear after 3 h of washing. The contractile effect of clonidine was very low (i.a. 0.13 0–0.17) and its relative affinity only 0.04. The contractions induced by clonidine were …er than those of norepinephrine and resembled those of oxymetazoline although they …re much weaker. The effect of clonidine in the dose range 1 10⁻⁸ 1 10⁻⁴ M was …cked by phentolamine 2.6 10⁻⁸ M.

… *Test of antagonists in the rabbit aorta.* Phentolamine 8.0 10⁻⁹ – 8.0 10⁻ M caused a …rallel displacement of the dose response curv of norepinephrine to the right without …ecting the slope (p 0.1) or the maximal response (p 0.25). The slope of the Schild plot …r phentolamine was not significantly different from unity (1.01 (0.81 1.21)) (n = 15 from …expts.). The pA calculated from the experiment was 7.59 (7.18–7.81) and K_B 7.4 10⁻⁸ M. … Tolazoline 3 10⁻⁸ 3 10⁻⁶ M caused a displacement of the dose response curves of …norepinephrine to the right without affecting the maximal responses (p 0.25). There was … significant effect on the slopes of the dose response curves by tolazoline ($p < 0.01$) …ecause the slopes became steeper. In spite of this the slope of the Schild plot was close to …unity (0.98 (0.78–1.17)) (18 from 6 expts.). The pA of tolazoline was 5.51 (5.6–5.85) and the apparent K_B 3.1 10⁻⁶ M.

Discussion

In the present work tw lines were used in the attempts to characterize α-receptors present in the studied organs. When agonists were tested, dose-response curves of the drugs were obtained and the relative affinity and intrinsic activity were taken as a primitive characterization of the drug-receptor interaction. When antagonists were tested, dose-response curves of norepinephrine were made in the bsence and presence of blocker and the data was analyzed by the Schild equation (Arunlakshana and Schild 1959).

Alpha-receptors in cholinergic neurons

The results obtained on the electrically stimulated terminal ileum were almost identical …th those obtained on proximal ileum, and they will therefore be discussed together.

Clonidine has been suggested to stimulate α-receptors in central and peripheral nervous tissues including the cholinergic neurons of the gastrointestinal tract (Kobinger and Walland 1972, Starke and Altman 1973, Bolme *et al.* 1974, Kobinger and Pichler 1975

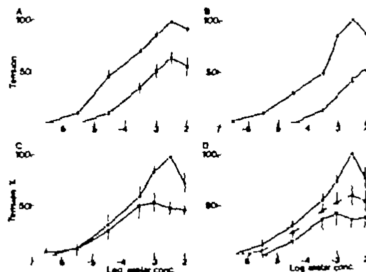


Fig. 9. Test of [illegible] atropinized guinea pig [illegible] ileum. Norepinephrine [illegible] as agonist in all [illegible]. Control response by [illegible]ephrine alone ●—●. A. Tolazoline 1 10^{-4} M □—□ (n 6). B. Phentolamine 2.7 10^{-6} M (n = 7). C. Phenoxybenzamine 1 10^{-6} M ○—○ ([illegible] D. [illegible] benzamine 3 10^{-7} M [illegible] dibenamine 3 10^{-7} M [illegible] (n = 6).

stimulation (p < 0.6) of the preparations (Fig. 6 C). The pA of phenylephrine was [illegible] low 4.20 (3.66–4.73), and K_B was estimated to be 6.3 10^{-4} M.

Test of agonists on the terminal ileum contractile response. In the presence of atropine (3.5 10^{-} M) and sotalol (5 10^{-} M) norepinephrine, epinephrine and phenylephrine induced strong contractions of the guinea pig terminal ileum. Under these conditions oxymetazoline (4 10^{-8}–4 10^{-4} M), clonidine (1 10^{-8}–1 10^{-} M) and dopamine (3 10 –3 10^{-4} M) induced only weak contractions (Fig. 7).

Non-cumulative dose response curves of the agonists were obtained from tests on the terminal ileum and the results are shown in Fig. 8, and in Table 1. The dose response curves were often unsymmetric. At high concentrations of norepinephrine and epinephrine inhibition of the dose response curves was often observed. Due to the unsymmetric shape of the dose response curves in the atropinized terminal ileum, conventional graphic methods were used in the analysis of the data instead of the logistic function model.

Epinephrine was slightly more potent than norepinephrine and its intrinsic activity did not significantly differ from that of norepinephrine. Phenylephrine was slightly more potent than norepinephrine but its intrinsic activity was less (0.62 (0.37–0.86)) (p [illegible]). Oxymetazoline was 40 times more potent than norepinephrine but despite of this its intrinsic activity was low (0.19 (0.08–0.30)) (p 0.01). Due to the high concentrations needed the maximal responses of dopamine and clonidine were probably not reached. The relative affinity of clonidine was estimated to be less than 0.2 and its intrinsic activity more than 0.22 (0.12–0.33). The relative affinity of dopamine was less than 0.07 and the intrinsic activity more than 0.40 (0.23–0.57).

Test of antagonists in the terminal ileum, inhibition of contractile response. Phentolamine (2.7 10^{-4} M) shifted the dose response curve of norepinephrine parallelly to the right although the maximal effect of norepinephrine was not reached after the blocking drug (Fig. 9 B). The pA of phentolamine calculated from the experiment was 7.13 (6.76–7.50) and the estimated dissociation constant 7.4 10 M. Tolazoline (1 10^{-4} M) also caused a parallel shift to the right of the dose response curve of norepinephrine (Fig. 9 A). The pA of tolazoline was 6.22 (5.83–6.60) and the estimated dissociation constant 6.3 10^{-7} M

...not fulfilled. Sanders et al. (1975) suggested that tolazoline was a weak α-adrenergic ...ist in rabbit aorta. It is possible that a weak intrinsic activity of tolazoline might have ...responsible for the observed increase in the slopes of the dose response curves of ...pinephrine.

...parison between α-receptors in cholinergic neurons and smooth muscle cells

...out clear-cut evidence for the existence of subtypes of α-receptors in the studied prepara-...s were derived from the tests with adrenergic agonists. There was a hundredfold ...erence between the relative affinities of clonidine on inhibition of electric stimulation of ...inal ileum and contracting the same organ after atropine. The difference between ...relative affinities of clonidine on electrically stimulated proximal ileum and rabbit ...a ...as of similar magnitude: about 160. In relation to norepinephrine, phenylephrine ...four times more potent in contracting the atropinized terminal ileum whereas it was ...ost completely ineffective in inhibiting contractions elicited by electric field stimulation ...he same organ. The drug actually was a competitive blocker to norepinephrine in this ...paration, ...hich indicated that phenylephrine had affinity but no activity at the ...drenoceptor of the neurons. The difference between the relative affinities of phenyleph-...e on electrically stimulated ileum and rabbit aorta was approximately 10. The drug ...owed maximal intrinsic activity in rabbit aorta, whereas it was only a weak agonist in ...um. The other studied agonists, i.e. oxymetazoline, epinephrine and dopamine did not ...ow any selective action on α-receptors located to neurons or smooth muscle cells.

Phentolamine was 55 fold more effective in blocking the α-receptor located in smooth ...uscle of guinea pig terminal ileum than in blocking the α-receptor of the cholinergic ...euron. The blockade induced by phentolamine on the α-receptor of the neurons might not ...e competitive. The slope of the Schild plot obtained from proximal ileum was far from ...nity and phentolamine diminished the maximal effect induced by norepinephrine.

Tolazoline was 8 times more potent in blocking ...-receptors in cholinergic neurons of ...uinea pig than in blocking α-receptors in the rabbit aorta. The potency of tolazoline to ...lock α-receptors in cholinergic neuron and smooth muscle cells of guinea pig terminal ...leum was of the same magnitude, however.

Phenoxybenzamine induced ... non-competitive blockade of the α-receptor located to smooth muscle of guinea pig terminal ileum but it was ineffective on the receptor of the cholinergic neurons, even though the same concentration and incubation time of the blocker was used in both tests.

Nomenclature of α-receptors

Subclasses of α-receptors might be named by their anatomical localization such as pre- or postsynaptic. There are certain disadvantages inherit with this nomenclature, however. Postsynaptic α-receptors could be localized in nervous tissue such as sympathetic ganglia (De Groat and Volle 1966) as well as in effector organs. Until the pharmacological properties of such or other α-receptors are compared with α-receptors of adrenergic or cholinergic nerves it is preferable not to refer to these receptors by their localization. Langer (1974) suggested that presynaptic α-receptors of adrenergic neurons should be

Vizi 1976). Oxymetazoline has also been suggested to have α-stimulatory properties in the rabbit intestine (Mujic and van Rossum 1965) and the rabbit aorta (Sanders et al 1975).

The experiments of the present study indicated that the tested agonists inhibited the electrically stimulated ileum by stimulating α-receptors. The agonists induced dose effects after β-receptors had been blocked by sotalol. Tolazoline acted as a competitive blocker against norepinephrine. This conclusion was reached since all criteria for competitive antagonism were fulfilled. The effects of the other tested agonists were also blocked by tolazoline, indicating that all agonists acted through stimulating α-receptors. The blocking properties of dibenamine also supported the theory that the adrenergic receptor of the cholinergic neuron was an α-receptor.

Recently some evidence has been presented that clonidine is capable of stimulating H_2-receptors (Karppanen and Westerman 1973, Csongrady and Kobinger 1974 and Andersson et al 1978). In this study there was no evidence that clonidine or oxymetazoline activated H_2-receptors in electrically stimulated proximal ileum since the responses to these drugs were not blocked by cimetidine. Dopamine was tenfold less potent than norepinephrine in the electrically stimulated terminal ileum. The receptor of the cholinergic neuron should therefore probably not be classified as dopaminergic.

Alpha-receptor in smooth muscle

Guinea-pig terminal ileum After blocking β-receptors with sotalol and cholinergic receptors with atropine the adrenergic agonists contracted the terminal ileum. Under these conditions it is probable that the responses were mediated through stimulation of receptors located to the smooth muscle cells.

It is probable that norepinephrine acted by stimulating α-receptors since both tolazoline and phentolamine caused parallel displacements of the dose response curve. The maximal effect of the agonist was not reached after the blockers. This may be explained by the presence of an inhibitory effect of norepinephrine at high concentrations. An inhibitory effect of catecholamines and related drugs, independent of α- or β-receptors, was present in denervated guinea pig proximal ileum when high concentrations were used (Wikberg 1977).

The blocking effect of phenoxybenzamine and dibenamine against norepinephrine indicates that the excitatory receptor in guinea pig proximal ileum should be classified as an α-receptor. The haloalkylamines induced a non-competitive type of receptor blockade since the dose response curves of the agonist were not at all shifted to the right.

Rabbit aorta The relative affinities of norepinephrine, epinephrine, phenylephrine and oxymetazoline on rabbit aorta found in this study were close to those reported by others on the same preparation (Bevan and Osher 1965, Furchgott 1970, Sanders et al 1975). The intrinsic activity and relative affinities of clonidine on rabbit aorta was similar to that found by Starke et al (1974) on rabbit pulmonary artery. In rabbit aorta clonidine probably stimulated α-receptors since the weak contractions were blocked by phentolamine.

It is concluded that phentolamine acted as a competitive blocker against norepinephrine since all criteria for competitive antagonism were fulfilled. Tolazoline probably also blocked the effect of norepinephrine competitively although all criteria for com   titive antagonism

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referred to as α_1-receptors while postsynaptic α-receptors should be referred to as α_2 receptors This suggestion was also notified by Drew (1977 a) According to the nomenclature it is suggested that α_1-receptors are defined as those α-receptors for which the relative potency is clonidine > phenylephrine. Furthermore α_2-receptors are defined as those for which the relative potency is phenylephrine > clonidine. By this definition receptors were present in the smooth muscle of guinea pig terminal ileum and in rabbit aorta α_1-Receptors were present in the cholinergic neurons of proximal and terminal guinea pig ileum.

I am indebted to Assistant Professo Rolf Andersson and Professor Lennart Lundholm for valuable criticism of this wo k. Financial support was provided by the S edish State Medical Research Council (04X-4498-04) and Magnus Bergvall Foundation

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Acta physiol. scand. 1978 103, 240–251
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Autoradiography on erythrokinesis and multihemoglobins in juvenile *Salmo salar* L. at various respiratory gas regimes

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Received 10 October 1977

Abstract

HÄRDIG J., L. Å OLSSON and L. B HÖGLUND *Autoradiography on erythrokinesis and multihemoglobins in juvenile* Salmo salar L. *at various respiratory gas regimes.* Acta physiol scand 1978 *103* 240–251

The kinetics of ythropoiesis in 5–6 month old *Salmo salar* L. was correlated to 4 combinations environment I PO_2 and pH/PCO_2. Given single doses of ^{59}Fe at the start of continuous flow bioassay the incorporation was examined in circulati g red blood cells (RBC) by autoradiography on blood smears The proportions of mmature labelled mmature nd labelled mature erythrocytes were calculated f samples tak   t intervals up to 52 days. Compared to control fish kept at a PO corresponding to % air saturation and pH/PCO t 7 6/8 pH units, mmHg, xygen depletion t 50 air saturation slowed the proliferation f RBC stem cells nd enhanced RBC maturation. *Ceteris paribus*, sustained hypercapnia at a PCO in the respiratory w ter raised to 23 mmHg (pH 7 1 7.2), stimulated proliferation but did not affect the outp t of mature RBC. Simultaneously lowered PO a d raised PCO_2 t the levels mentioned abo   bviously stressed the f sh, as the effect of lowered PO *per se* was not manifested. Radioactivity was traced t all electrophoretically separable protein fractions from RBC hemolysates. The relevance of blood physiological criteria f probing th f t ess or well being of fish in environmental respiratory stress cond tions has been discussed

An autoradiographic approach for studying erythrokinetical events in teleosts experimentally exposed to well defined environmental changes, does not seem to have been made previously The present study was initiated in order to determine if the exposure for about 7 weeks to different respiratory gas regimes effects the "multihemoglobin pattern" (Koch 197?) and erythropoiesis in young salmon. On the whole, the present knowledge of the kinetics of fish erythropoiesis is relatively scanty As in mammals the hemoglobin content and hematocrit is supposed to increase in the blood of fish due to environmental hypoxia (Phillips 1947 Chiba 1965 Randall 1970) At least in salmonids these blood parameters and the total number of erythrocytes also increase at rising water temperatures (Dewilde and Houston 1967 Miles and Smith 1968) Powers (1932) gave evidence in *Ictalurus punctatus* for an increased number of circulating red blood cells (RBC) in response to oxygen depletion combined with a slight increase in carbon dioxide tension in the respiratory water

[illegible] of serum from anemic or hypoxic fish stimulates erythropoiesis in the blue [illegible] (*Trichogaster trichopterus*) (Zanjani *et al.* 1969). In the same species Yu *et al.* [illegible] demonstrated that injected radioactive iron is stored in the spleen, kidney and liver [illegible], which is a supply of iron used when erythropoiesis is stimulated. Thus [illegible]ropoiesis in fish seems to be controlled by similar mechanisms as found in mammals [illegible] *et al.* 1969).

[illegible] new circulating RBC appear as immature round erythroblasts which can be [illegible] microscopically from more elongated mature erythrocytes (Topf 1955). Härdig [illegible] has discussed this matter more closely along with studies on hemoglobin synthesis [illegible] salmon (*Salmo salar*) based on observations of the incorporation of radioactive [illegible]

[illegible] attempt has been made in the present study to apply continuous flow bioassays [illegible] and Härdig 1969, Börjeson and Höglund 1976) in order to correlate the kinetics [illegible] RBC production and RBC maturation in juvenile Baltic salmon (*Salmo salar*) to four [illegible] combinations of PO[illegible] and PCO_2 in the respiratory water. At the start of the [illegible] lasting for 52 days each specimen was injected intraperitoneally with a single dose [illegible] ^{59}Fe. The incorporation of radionuclide into immature and mature red cells has been [illegible] on autoradiograms of blood smears from 5 to 7 specimens exposed to each [illegible] for various lengths of time (*cf* p. 242).

[illegible] simultaneously the incorporation of radioactive iron into the electrophoretically separ[illegible] fractions of RBC haemolysates was checked on fish from all test groups.

Material and methods

[illegible] *fish.* In all 192 five to six month old salmon parr (*Salmo salar* L.) with [illegible] mean weight and S.D. of [illegible] g (range 2–9 g) [illegible] used in the tests. The animal material [illegible] supplied by the Fishery Board of [illegible] from the hatchery and rearing plant at Älvkarleby at the River Dalälven near its outlet in [illegible] the [illegible] It represented [illegible] population of Baltic salmon derived from eggs of many females fertilized [illegible] miscellaneous males. Before used in the experiments all fish were preacclimated for 2 weeks in our [illegible] by running aerated Uppsala tap water equal to the control conditions in the assays. In [illegible] they were fed daily even during the tests with salmon starter fodder pellets (Astra Ewos, [illegible], Sweden).

Water quality. The original feed water [illegible] Uppsala tap water. It is [illegible] water from an esker [illegible] in some parts through areas with limestone deposits. It is poor in oxygen and has [illegible] fairly [illegible] ion composition. Ca^{2+} 2.0–2.2 mmol/l, Mg^{2+} 0.5–0.6 mmol/l, and HCO_3^- about 5 mmol/l are the [illegible] constituents (Höglund *et al.* 1974). About 50 fish were placed in each of 4 continuous flow test [illegible], 5–7 specimens from each batch were removed at intervals and then killed. Thus specimens [illegible] kept in the test troughs for at most 52 days. Test group I being the "control" was run equal to the [illegible] environment in [illegible] oxygenated tap water (90 [illegible] air saturation) [illegible] moderately high [illegible] dioxide tension (about 8 mmHg at pH 7.6). In the 3 other vessels the following combinations of [illegible] gas parameters were established: lowered oxygen content and moderate carbon dioxide tension in Test group II, sufficient oxygen and raised PCO_2 in Test group III, lowered PO_2 as well as raised PCO_2 in Group IV as shown in Table I. PCO_2, PO_2 and pH [illegible] maintained constant and controlled daily for the [illegible] period of 52 days [illegible] each test vessel containing 60 l and drained at the rate of 550–650 [illegible]. A definite PO_2 was secured by the accurate mixing of non-aerated and air-saturated tap water. Carbon dioxide over-saturation was kept at the desired fixed PCO_2 by the continuous addition of the appropriate amounts of hydrochloric acid to the actual drainage of bicarbonate-rich supply water (Höglund [illegible] Höglund and Härdig 1969). The oxygen content [illegible] determined by Winkler titrations as modified by Carpenter (1965). The HCO_3^--alkalinity [illegible] measured titrometrically according to Berger (see Karlgren

TABLE I The 4 combinations of respiratory gas regimes in the ambient water used in continual bioassays lasting for 52 days. Daily determinations of O_2 in per cent air saturation, PCO_2 mmHg and pH are given as means and range.

Test group	I			II			III			IV		
	Moderate PCO_2						High PCO_2					
	PO_2 next to air saturation			PO_2 reduced			PO_2 next to air saturation			PO_2 reduced		
	O_2 % of air sat.	PCO_2 mmHg	pH	O_2 % of air sat.	PCO_2 mmHg	pH	O_2 % of air sat.	PCO_2 mmHg	pH	O_2 % of air sat.	PCO_2 mmHg	pH
Mean	89	8	7.6	49	8	7.6	87	[illegible]3	7.1	49	23	7[illegible]
Range	85–91	8.0–8.[illegible]	7.5–7.6	44–52	8.0–8.[illegible]	7.5–7.6	84–90	19.8–[illegible]6.7	7.0–7.2	47–52	20.3–2[illegible]	[illegible]

196[illegible]) and PCO_2 calculated on the basis of alkalinity, pH and temperature using the constants applied [illegible] Höglund (1961).

Blood sampling and autoradiographs. Just before starting the exposures (day 0 in the bioassays) [illegible] fish was given one standard dose of 1 μCi ^{59}Fe by i.p. injection (0.1 1 μg Fe as ferric citrate containing 1 [illegible] sodium citrate made isotonic with NaCl, Amersham England 1971, code IES[illegible]P). Blood was sampled after the exposures of 3, 6, 10, 15, 20, 30, 41 and 52 days for autoradiography on blood smears.

Electrophoresis. At the intervals mentioned above blood was also taken from two fish from each group for electrophoretic separation of "multihemoglobins" (*cf.* Koch 1972). The erythrocytes were washed in 0.9 [illegible] NaCl, hemolysed in distilled water and electrophoresis was carried out on starch [illegible] using Tris/HCL buffer at pH 8.3 as described by Koch *et al.* (1964). The prepared electropherograms were pressed against Ilford Red Seal X-ray films and exposed for 15 days at 0–4 C and then developed in Kodak D19B.

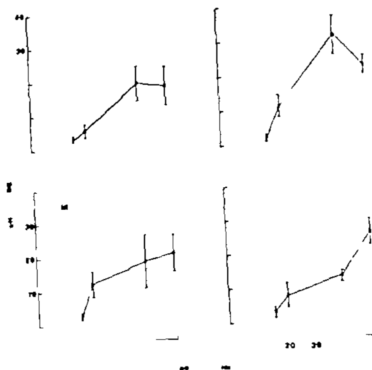
Light microscopy and the classification of RBC as to incorporation of radioactive iron and the degree of maturation. The preparations of blood smears were examined under light microscope at the magnification of 720 times. The incorporation of radioactive iron and the classification of red cells as "immature" [illegible] cells of Classes 1–4 according to Härdig (1978) dealing with the same primary observations [illegible] Accordingly the remaining morphological types of RBC belonging to Classes 5–6 (Härdig 1978) are [illegible] here as "mature" RBC. The percentages of immature RBC of all cells examined have been calculated as well as the proportions of labelled cells in the groups scored as immature and mature erythrocytes. The calculations are based on approximately 100 RBC examined from each blood smear. Blood samples were taken from seven fish at the exposure times chosen in Test groups I and III and from 5 fish at the same dates in Test groups II and IV. Two blood smear preparations were obtained from each fish.

Statistical treatment. For the sake of simplicity the regressions calculated for ascending parts of the curves (Day 15–41) in Fig. 1–3 (Table II) were considered to be linear. In Fig. 2 comparisons were made within experimental groups. Otherwise comparisons between test groups and control group have been accomplished regarding pairs of experimental series. All significance levels have been determined with Student's t-test.

Results

A The categories of RBC and hematological parameters studied

In young salmon immature RBC delivered to the circulation exhibit continuing morpho[illegible] metric changes. In the present study the RBC were categorised as belonging to two age groups, *viz.* an earlier one without and a later one with iron uptake since the start of the assays and the simultaneous administration of ^{59}Fe. Thus during the present exposures to



1 Changes in the proportion of labelled red blood cells (mean values and S.D. for n = 5–7) in per cent of RBC examined during the course of exposures to four respiratory media (see Table I). Each test fish given one single dose of ^{59}Fe at zero time. Constants for regression lines calculated on the same values covered from Day 15 until Day 41 are given in Table II

four respiratory gas media, the RBC—if incorporating the radionuclide—after entering the blood stream, pass through the unlabelled and then through the labelled maturation compartment into the labelled mature compartment.

The following four categories of RBC have been dealt with 1) labelled RBC (Fig. 1); 2) immature RBC (Fig. 2); 3) immature labelled RBC (Fig. ?); 4) mature labelled RBC (Fig. 3). The levels of the fractions of these categories have been followed after the administration of ^{59}Fe in order to obtain data as to the sizes and transit times of the two maturation compartments discerned. The flow out of the maturation compartment was measured by the changes of the levels of mature labelled RBC with time.

The increase of the RBC pool in the maturation compartments is caused either by a new formation, inflow from RBC depots and/or by elongated transit time, i.e. a more or less temporarily delayed maturation.

1. *Erythropoietic events in each respiratory environment tested*

Comments on the present control condition of Test group I. In the control fish (Test group I in Table I) kept in water with high oxygen (corresponding to 90% air saturation)

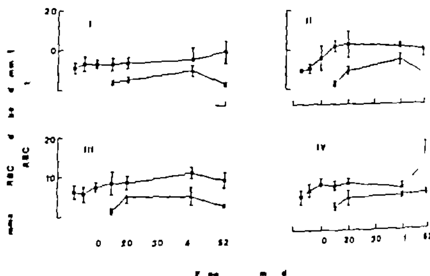


Fig. 2. Changes in the percentages (mean values and S.D for n = 5–7) of immature RBC (■) of all cells examined at 7 occasions and changes in the percentages of labelled immature RBC (▲) studied after variously long exposures to each of 4 combinations of oxygen content and carbon dioxide tension in ambient water (Table I). At the start of the tests the fish were given single doses of ⁵⁹Fe.
The increase in the percentage of immature RBC as compared with that for Day 3 was determined within each group. Significance levels were $0.01 < p < 0.05$, $0.001 < p < 0.01$ and $p < 0.001$.

and low carbon dioxide tension (PCO 8 mmHg), after a delay until Day 15 the percentage of labelled erythrocytes increased from 3 per cent at Day 15 to 19 per cent at Day 41 and then it levelled off (Fig. 1 Table II)

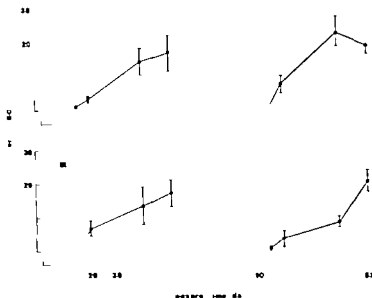
The proportion of immature RBC, (*i.e* the sum of labelled and unlabelled cells as per cent of all cells examined) did not change significantly during the test period (Fig. 2). The pool size of labelled immature RBC on the other hand, increased during the part of the test lasting from Day 15 to Day 41 After that it declined until the end of the test at Day 52 (Fig. 2) This final decrease most likely was due to diminishing contents of ⁵⁹Fe in the body iron stores.

With the limitations discussed on p 247 the data in Fig. 2 may permit by extrapolation an approximate estimate of transit time for labelled immature RBC. In the Test group I it amounts to about 20 days. The constants of the regression lines presented in Table II can be used to estimate erythropoietic activity measured as the increase in percentage of mature labelled RBC per day This will give a numerical value of 0.51 valid for the animals in Group I

2. *Activation by lowered PO in Test group II* In the fish kept in water with a reduced oxygen content, the fraction of labelled RBC increased at a significantly higher rate than in the controls (Fig. 1 Table II)

The proportion of immature RBC increased rapidly from Day 3 to Day 20 and then it levelled off Values significantly higher than in the control group were attained within the period from Day 15 to Day 41 (upper curve in Fig. 2)

At the examinations made after 20 and 41 days the level of labelled immature RBC was also significantly higher than in the control group and a decrease from Day 41 to 52



3 Changes in the proportions (mean values and ± S.D. for n = 5–7) of labelled mature RBC in young salmon after given a single dose of [59]Fe at the start of 4 assays with different combinations of oxygen content and carbon dioxide tension in the respiratory water as shown in Table I. Constants for regression lines fitted on the observations from Day 15 until Day 41 are given in Table II.

...n also seen here, presumably for reasons discussed above (p. 244). No exact determination of transit time could be done because the increasing proportion of immature RBC. ...t as the total amount of immature cells was approximately the same Day 41 and 52, the slope of the line for labelled immature RBC for this part of the assay has been used to estimate the transit time for labelled immature RBC. Then also in this case it will amount to about 20 days (*cf* Test group I). If this holds good the observed increase in immature RBC must be the result of an enhanced output of RBC, which will also explain the observed high level of mature labelled RBC, nearly reaching 20 per cent at Day 41 (Fig. 3).

As compared to the controls the erythropoietic activity was significantly enhanced in the Test group II giving an increase in the percentage of mature labelled RBC per day of 0.80 (Fig. 3, Table II). Thus at pH/PCO 7.6/8 pH units/mmHg an oxygen depletion from 90 to 50 per cent air saturation stimulated the erythropoietic activity in the present yearlings of *Salmo salar* L., evidently by proliferating RBC stem cells as well as enhancing RBC maturation. This was manifested within a period of 20 days since the fish were submitted to the new environment.

3 *Effects of high PCO in Test group III* In the assay with carbon dioxide supersaturated water there was a significant increase in the labelled cells during the whole test period (Fig. 1, Table II). The regression line for the changes in the proportion of labelled RBC with time are not significantly separated from each other in Test groups I and III (see Table II). Thus no effects of the high PCO was obtained in this parameter

TABLE II Statistics for the correlation between assay time in days (from Day 15 until Day 41) and percentages of A) labelled RBC (Fig. 1) and B) labelled mature RBC (Fig. 3).

	x days	y per cent	Test group	n	b[a]	r[b]
A	Assay time	Labelled cells	I	17	0.63	0.94
			II	14	1.07	0.95
			III	19	0.55	0.74**
			IV	14	0.35	0.85
B	Assay time	Labelled mature cells	I	16	0.51	0.95
			II	12	0.80	0.95**
			III	16	0.44	0.81
			IV	12	0.28	0.80*

[a] The regression coefficients (b) for Test groups II, III and IV have been compared with that in the control in Group I using t test.
[b] Correlation coefficient r
The significance levels were * $0.01 < p < 0.05$, ** $0.001 < p < 0.01$ and *** $p < 0.001$

The proportion of immature RBC increased with time as shown in Fig. 2 (III, upper curve) and the value for Day 41 was significantly higher than in the control group ($0.001 < p < 0.01$).

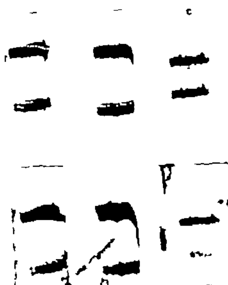
The proportion of labelled immature RBC changed as shown in Fig. 2 (III, lower curve) but no significant differences occurred when compared to the control group. Whether the transit time for immature RBC through the labelled maturation compartment was elongated compared to the control group cannot be exactly estimated from the present data. By extrapolation in Fig. 2 (III), however, it may be approximated in Test group III to about 3 days.

The erythropoietic activity, measured as the increase in the percentage of mature RBC per day, amounted to 0.44 which is not statistically different from the value found in Test group I (Table II).

Neither were the increasing levels of immature RBC as compared to the control group (Fig. 2) accompanied by a significantly changed output of mature RBC (Fig. 3, Table II). In this group the high PCO probably acted on the proliferation of the stem cells in order to increase the rate of erythropoiesis. Probably by elongating the RBC maturation time the net output of mature cells is not higher than in the control group. Instead accumulation of unlabelled immature RBC may occur.

4 *Combined effects of lowered PO and high PCO in Test group IV*. In Test group IV there was a significant increase in labelled RBC over the whole test period (Fig. 1, Table II). In this case the regression for the increase in the proportion of labelled RBC from Day 15 to Day 41 was significantly lower than in the control group (Table II).

The level of immature RBC increased as shown in Fig. 2 (IV) and a significantly higher value was found for this parameter at Day 10, 15, 20 and 52 when compared to Day 1. From Day 41 to 52 a significant increase was also obtained $0.01 < p < 0.05$. Comparing Test group IV with the control group (I) the parameter dealt with in the upper curves of Fig. 2 differs significantly between groups for Day 52 only ($0.01 < p < 0.05$).



4 Electropherograms (above) and autoradiograms (below) of haemolysates from control fish belonging to Test group I in Table I. Time after the administration of ^{59}Fe was 15, 20, and 41 days in A, B and C, respectively. Essentially the same result was arrived at regarding Test groups II, III and IV for all days of observation.

Contrary to the results in Test groups I–III no decrease in the level of labelled immature RBC was found in Test group IV from Day 41 to Day 52. The turnover of iron thus was lower in the latter group and the transit time for immature RBC prolonged.

As presented in Fig. 3 (IV) and Table II also the output of labelled mature RBC was essentially lowered in this group until Day 41. Then a rapid increase in the percentage of labelled mature RBC started. The estimate for erythropoietic activity from Day 15 to Day 41 gives a numerical value of about 0.28 per cent increase in labelled mature RBC per day (Table II). This evidently also points to an impaired erythropoiesis.

Incorporation of ^{59}Fe and the multihemoglobin pattern

The autoradiograms for the electropherograms in Fig. 4 shows that ^{59}Fe was present in all haemolysate fractions from Day 15 until Day 52, i.e. from the time when radioactive iron became detectable within the red cells.

Discussion

A. *Shortcomings in methodological respects*

The growth rates of test fish was not examined and the changes in blood volume could not be controlled. There was a great variation in fish body weights but no correlation was found between the amount of immature RBC and fish weight in any group at any day of sampling. The estimations made here of pool sizes and transit times regarding the two maturation compartments can only be done provided that the new formation of RBC and the maturation rate are constant during one experiment which apparently is not true.

The experiments were designed to be cohort labelling (Berlin 1964). The percentages of labelled mature RBC were thus expected to reach a plateau and then to decline. As seen in Figs. 1 and 3 this did not occur. Inadequate amounts of administrated ^{59}Fe, too short

duration of the tests as well as increased erythropoietic rate and/or RBC volume may h[illegible] contributed to this discrepancy

B *Starting point for the incorporation of radioactive iron*

Härdig (1978) found that the iron uptake of RBC was completed before reaching the mature stage. In the present study no incorporation of the injected iron could be detected in RBC considered to be mature at the time for the administration of radioactive iron. This indicates that mature cells did not start new hemoglobin synthesis in response to the present hypoxia or other environmental conditions used in the assays.

From fish exposed for 3 and 6 days in either test group none, and after 10 days exposure still very few labelled cells appeared in the autoradiographs. Thus labelled cells were falling during early parts of the tests as shown by the presentations in Table II and Fig. 1–3 The lag phases in the appearance of labelled RBC probably reflect the time needed for the incorporation of administered radionuclide in the iron pools and to reach [illegible] grain counts above the labelling level chosen (*cf* p 242)

C *Morphology and life span of fish RBC*

The oldest morphological type, or senile RBC discerned in the carp by Topf (1955) could not be recognized here, but all other stages in the development series of RBC described by him were identified (Härdig 1978) The absence of senile RBC might be explained by the postulate that the RBC life span exceeds the age of the fish material and also the exposure time used in the present study Altland and Brace (1962) report that the mean live span of turtle RBC probably is 600–800 days as compared to 700–1 400 days for tailess frogs (Anura)

D *Evaluation of erythrokinetic responses induced*

It appears from the present results in Fig. 2, Test groups II III and IV in comparison to the Control group I that some effective mechanism(s) exist which act upon the erythron in response to the respiratory gas parameters PO_2, PCO_2 and/or pH This indicates that the proliferation of RBC stem cells were stimulated

The erythropoiesis was activated within a few weeks by the present reduction of oxygen content in the respiratory water which caused an increase in the RBC maturation pool and enhanced the output of mature RBC (Fig. 2 and 3 Table II) This verifies earlier findings *i.e* by Zanjani *et al* (1969) in another species of fish (*Trichogaster trichopterus*).

The carbon dioxide oversaturation in Test group III did not change the output of mature RBC (Fig. 3 Table II) Rather a retardation of RBC maturation may occur This should be considered in connection to the findings by Börjeson and Höglund (1976), that sustained CO_2 oversaturation almost equal to that in the present test conditions of Group III (*cf* Höglund and Härdig 1969 Höglund and Börjeson 1971 Höglund and Persson 1971) causes a pronounced Root effect which in young *Salmo salar* is transient and parallel to the acidosis initially caused by the diffusion of carbon dioxide from the environment into the blood. By compensatory acid-base reguation it fades out within 30 [illegible] (Börjeson and Höglund 1975, 1976, Börjeson 1976, 1977)

environment in Test group IV acted negatively upon the fish. The transit time for ure RBC was elongated and the output of mature RBC lowered. This is presumably disturbed metabolism on account of starvation and initial strain. It is known from mals, that an inadequate metabolic environment elongates the maturation time of Harris and Kellermeyer 1972). Zanjani *et al.* (1969) also report that in the blue gourami (*Trichogaster trichopterus*) the erythropoiesis is inhibited by starvation. Now in the case young salmon in Test group IV the specimens did not feed well until the fourth or week of the assay.

Implications of the present autoradiography on multihemoglobins

Electrophoretic pattern of so called multihemoglobins in salmon (Koch *et al.* 1964) is to depend on fish size, age, race and breed or physiological condition (Hashimoto and Matsuura 1962, Wilkins 1968, Wilkins and Iles 1968, Koch 1972). That is why electrophoreses on blood haemolysates were performed to possibly find correlations with the respiratory water qualities chosen. However the present respiratory stress conditions did not affect the "multihemoglobin" pattern in the young *Salmo salar* tested. Thus, our observations fully support the view expressed by Giles and Vanstone (1976) who were not able to induce changes in the pattern of multiple hemoglobins from coho salmon (*Oncorhynchus kisutch*) fry and pre-smolts by varying PO_2.

The intention was also to identify the biochemical background to the electrophoretic separation, which though using various electrophoretic and gel filtration techniques has so far now been unsuccessful. As ^{59}Fe incorporated in RBC presumably is bound to heme proteins, it cannot be decided to what extent the recorded radioactivity is due to hemoglobins or other ferroproteins such as cytochromes, catalases or peroxidases. Whether or not the salmon "multihemoglobins" are correlated with fish development and maturation of RBC could not be determined, as radioactivity appeared in all fractions separated from day 15 and onwards.

Thus, to summarize, the diagnostic value of changed "multihemoglobin" pattern in fish is questioned. The physiological emphasis and correlation to erythropoiesis of these protein fractions are still obscure.

Haematology in testing health condition in fish

Sprague (1976) criticizing sublethal tests of pollutants on fish referring to McKim *et al.* (1970) points out that the effects of copper on five blood parameters in *Salvelinus fontinalis* were either transient during 1-year exposures or less sensitive than effects on reproduction, growth and survival. This and other reviewed facts led Sprague (1976) to the general conclusion that laboratory blood physiological tests have yielded disappointingly few water quality criteria concerning the large number of investigations.

The main result of the present tests supports this view. A general test (like the sedimentation rate reaction in human medical diagnostics) to apply blood parameters in determining the state of physiological efficiency, injury or well being of fish is still missing.

[3] This investigation was financially supported by the Swedish Natural Science Research Council (grant No. B2173-010) and from the C. Groschinsky, Helge Ax:son Johnson, E. and Oscar Ahrén and O. E. Nycander Foundations and the Faculty of Mathematics and Science, University of Uppsala.

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Acta physiol. scand. 1978. 103. 252-262

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Effects of isoprenaline and cooling on histamine induced changes of capillary permeability in the rat hindquarter vascular bed

By

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Received 25 November 1977

Abstract

RIPPE, B and G J GREGA. *Effects of isoprenaline and cooling on histamine induced changes of capillary permeability in the rat hindquarter vascular bed* Acta physiol. scand. 1978 *103* 252-262.

Histamine infused intra arterially into artificially perfused, maximally dilated rat hindquarters markedly increased fluid filtration and CFC but had essentially no effect on the diffusion capacity to small molecules. Isoprenaline largely prevented the increase in fluid filtration and CFC if infused prior to the start of the histamine infusion and if infused after the start of the histamine infusion, promptly reduced fluid filtration and CFC to near control levels. Additionally it was noted that severe cooling of the perfusate largely prevented the marked increase in fluid filtration and CFC by histamine. This antagonism of histamine induced increases in macromolecular permeability represents a direct action of isoprenaline on the microvascular membrane which effectively counteracts that of histamine. The data also suggest that the large pores created by histamine are different from the large pore through which macromolecules normally transverse the microvascular membrane and that catecholamines may exert a regulatory function in the control of microvascular permeability to macromolecules in pathophysiological states associated with massive histamine release

Although the concept that the sympathetic nervous system and the circulating neurohormones adrenaline and noradrenaline may affect microvascular permeability to both micro- and macromolecules was proposed decades ago there followed little convincing evidence to support this hypothesis. These early studies (Engel 1941 Asher and Jost 1914) purporting that the sympathetics affect microvascular permeability were subjected to severe criticism (Chambers and Zweifach 1947 Danielli and Stock 1946) as the experimental approach to the problem was indirect and the investigators often failed to consider changes in hemodynamics and Starling capillary forces in the interpretation of their results. Attempts to substantiate these conclusions in better controlled studies were uniformly unsuccessful (Cheng 1949) that is, no measurable effect of the sympathetics on capillary permeability could be demonstrated. Subsequently it became universally accepted that normal microvascular permeability including that to macromolecules, was not under the influence of the sympathetic nervous system or circulating catecholamines (Folkow 1955).

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...ecent years some evidence has accumulated suggesting that in certain vascular beds, ...er certain conditions, this generalization may not be true. In canine adipose tissue, ...ample, sympathetic nerve stimulation increases microvascular membrane permeability ...g and Rosell 1967 Rosell, Intaglietta and Chisholm 1974 Linde and Gainer 1974 Chisholm and Rosell 1974). The possibility that sympathetic stimulation in this bed causes the liberation of histamine-like substances has not been excluded. Hence, ...creased microvascular permeability in adipose tissue in response to nerve stimulation ...y represent an indirect rather than a direct effect of the sympathetics. There is no ...nce that sympathetic stimulation increases microvascular permeability in other canine ...lar beds.

...has, however recently been demonstrated that the massive protein efflux caused by ...ntra-arterial infusion of histamine into the canine forelimb can be largely prevented by systemic hypotension (Marciniak *et al.* 1977) or alternatively by a simultaneous intra-...ial infusion of noradrenaline or isopropylnoradrenaline (isoprenaline) (Marciniak *et al.* ...). This antagonism of histamine protein efflux by prior systemic hypotension or by ...] catecholamine infusions could not be attributable simply to decreases in blood flow ...t also occurred during constant flow conditions. Evidently the sympathetic neuro-...mones could reduce the microvascular transfer of macromolecules under conditions ...n the permeability to macromolecules in canine forelimb skin and skeletal muscle has ...en greatly increased by histamine. However a reduction in perfused surface area could ...tribute to this effect and the extent of such a contribution remains to be determined. ...n ...iew of the possible importance of catecholamines as physiological antagonists of ...stamine and bradykinin (Maciejko *et al.* 1977) in the microcirculation, it was decided to ...dy in more detail the histamine-catecholamine microvascular permeability interaction ...der conditions when both diffusion and convection are simultaneously followed, utilizing ...n artificially perfused isolated rat hindquarter preparation as the test organ. One major ...m of this study was to determine if the antagonistic action of catecholamines on the histamine-induced increase in protein efflux reflected a direct action on the microvascular ...embrane alone or if also decreases in perfused surface area contributed importantly to this ...fect. It was further considered important to determine if this was a species-dependent ...fect as earlier studies were all performed on dogs.

Methods

[illegible] and technical arrangements. Experiments were performed on the isolated hindquarters from ...3 male Wistar albino rats, weighing between 270 and 380 g. The hindquarters [illegible] arranged for the "[illegible]" method (*cf.* Pappenheimer and Soto Rivera 1948, [illegible] *et al.* 1974) and for [illegible] colori-metric on line" modification of the single injection indicator diffusion method (Rippe and Haraldsson 1976, 1977).

The [illegible] rat hindquarter preparation has been described elsewhere (Folkow *et al.* 1974). Briefly, the tail artery [illegible] cannulated during ether anesthesia for continuous mean arterial pressure (P_a) recordings after which the animal was further anesthetized with Nembutal [illegible] 3 mg/100 g, and eviscerated. The abdominal aorta and the inferior caval vein were freed from the level of the renal vessels to the [illegible] lumbar ones. Then the hindquarters were isolated just proximal to these latter vessels by mass ligatures and the spinal channel and bone marrow were plugged with cotton wool soaked in silicone. Tail and paws were excluded by tight ligatures, so that the preparation consisted mainly of skeletal muscle (70–75 %).

After heparinization the abdominal aorta was cannulated and connected to an artificial perfusion

system, driven by a Harvard perfusion pump. The caval vein was connected to a venous outflow cannula while one of the renal veins was used for continuous venous pressure recordings (P_V). The entire isolated hindquarter preparation was then placed on a balance plate, connected to a strain gauge for continuous weight recordings. Finally the venous outflow cannula was coupled in series with a pH electrode and a densitometer system for simultaneous recording of Cardio-Green albumin and Cr-EDTA. The arterial inflow cannula was side-branched to a step dispenser syringe for injection of the mentioned indicators, for measurements of capillary diffusion events (*cf* Rippe and Stage 1978). The level of the venous outflow cannula, *i.e.* the one distal to the densitometer system, could be adjusted to any desired level to set P_V. Flow and P_A could be controlled by adjusting the setting of the perfusion pump. Tissue weight, P_A and P_V were continuously monitored on a Grass polygraph, while pH and the dye dilution curves were recorded on a potentiometer writer.

Four per cent dextran (Macrodex® MV about 70 000 supplied by AB Pharmacia, Sweden) in Tyrode was used as perfusate to which was added either horse serum (Normal Serum, SBL, Sweden) or bovine albumin (Fraction V Powder Sigma Chemical Company USA), 6 g/l. The perfusate was gassed with a mixture of 97 % O_2 and 3 % CO_2 and kept at 38 C. Perfusion flow was kept constant at 11-14 ml/min 100 g of tissue throughout the expt. Maximal vasodilatation was maintained during the expt by means of slow infusion of papaverine.

Capillary filtration and diffusion were continuously followed in these expts. Capillary hydraulic conductivity was determined as capillary filtration coefficient (CFC) (*cf* Eliassen *et al.* 1974). Large molecule permeability affecting the colloid osmotic pressure difference across the capillary walls, could be followed by the consequent changes in capillary fluid equilibrium, which caused net weight changes when the hydrostatic capillary pressure was kept constant. Small molecular permeability was assessed by measuring PS for Cr EDTA by a colorimetric modification of the single injection indicator diffusion method (Rippe and Stage 1978).

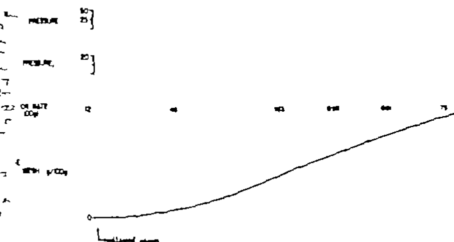
Experimental procedure

1 *Action of isoprenaline on the histamine stimulated hindquarter vascular bed.* Initially CFC and PS for Cr EDTA were measured 2–3 times while the hindquarter vascular bed was kept at complete isogravimetric state for at least 15 min. Histamine, 30–60 µg/ml of perfusate, was then infused into the preparation without changing flow or P_A in the already maximally dilated vascular bed. The concentrations of histamine used in this study were kept well above 15–20 µg/ml, which was tested out to be the concentration required to obtain definitely maximal increases in microvascular permeability. This relatively low responsiveness of the rat to histamine, compared with some other species, is in agreement with earlier studies (*e.g.* Fox and Wyland 1976). All 11 animals responded to histamine but only 6 with pronounced increases in CFC and with marked net outflux of fluid causing considerable weight increase. To these six preparations were maximal doses of isoprenaline 50–70 µg/ml of perfusate (1 4–1.9 × 10^{-4} M) were infused about 10 min after the start of the histamine infusion, so that the two drugs were now supplied simultaneously to the preparation. CFC, capillary fluid balance and PS for Cr EDTA were continuously followed during these expts (*cf* Results).

2 *Action of histamine on the isoprenaline pretreated hindquarter vascular bed.* Another four animals, which in other respects were treated in exactly the same way as the above mentioned ones, were infused with isoprenaline, going on for some 10 min before the infusion of histamine was started in parallel. Capillary permeability characteristics were followed as described above.

3 *Approximate dose levels of isoprenaline required to suppress histamine induced permeability changes.* In order to obtain a rough estimation of the dose levels of isoprenaline required to partially or completely abolish the histamine induced changes in microvascular permeability the following procedures were employed. To four hindquarters perfused with dextran and "aged" horse serum (see Results) supramaximal histamine infusions were first given for 8 min and the permeability changes were followed with CFC measurements. Subsequently an infusion of isoprenaline was given simultaneously with the histamine infusion in increasing concentrations, from 0.5 to 1 000 µg/ml perfusate (1 4 × 10^{-9} – 2.7 × 10^{-6} M) in three steps during a total period of 15–20 min. At every dose level CFC was measured and could be compared to the values obtained during the pure histamine infusion period. All measurements were performed within 25 min after the start of the histamine infusion, because in some experiments in which only histamine was infused a spontaneous decrease of CFC could be seen at this time (unpublished results).

4 *Action of histamine on the cooled hindquarter vascular bed.* In 6 animals CFC was first measured during control at normal tissue temperature (36–37°C). The tissue temperature was then reduced to between 10 and 15°C by means of cooling the perfusate to around 5°C. After CFC measurements at 10–15°C the cooled



1. The effect of supramaximal doses of histamine (30 µg/ml min 100 g) on the capillary fluid filtration, represented by changes in tissue weight in the maximally dilated hindquarter vascular bed. [illegible] recording is taken from preparation where CFC measurements were made after the time period [illegible] in the Figure. The filtration rate measured 6–8 min after the start of the histamine infusion as well as CFC values obtained 15 min later are approximately the same as those in the expt. shown in Fig. 3 [illegible] the isoprenaline infusion started.

[illegible] preparation was kept at isogravimetry for some 10 min after which histamine, 30–60 µg/ml [illegible] was infused as described above. The histamine infusion was continued for 20 min and changes [in] CFC and capillary fluid balance were registered. For an additional 15 min after stopping the histamine [infu]sion the preparation was kept at 10–15°C, after which it was rewarmed to 37°C by means of increasing [the] perfusion temperature to 38 C. After control CFC measurements and a new period of isogravimetry [hist]amine was again infused into the preparation in exactly the same dose as earlier and the effects on CFC [and] tissue weight were studied.

Results

Actions of isoprenaline on the histamine stimulated hindquarter vascular bed. In the 6 [s]elected hindquarters (see above) histamine caused an increase in CFC from 0.0483 ± 0.0030 ml/min mmHg 100 g (mean S.E.) at control, to 0.1315 ± 0.0019 ml/min mmHg 100 [g]. Under the influence of histamine the preparations also started to gain weight, i.e. to [filt]rate, from a state of complete isogravimetry and this usually occurred within 2 min from the start of the infusion. In the 6 preparations mean filtration rate amounted to 0.86 ± 0.18 ml/min 100 g 5–8 min after the start of the infusion (see Fig. 1 and 2).

During the histamine-isoprenaline infusion CFC rapidly returned to near control 0.0510 ± 0.0033 ml/min mmHg 100 g (p 0.001) and the filtration rate decreased significantly to 0.18 0.03 ml/min 100 g within 5–10 min (p 0.01) (see Fig. 2 and 3).

After stopping the isoprenaline-histamine infusion the preparations seemed to be abso[lu]tely refractory to histamine for the following 30–60 min and relatively refractory to histamine for another 1 or 2 h. PS for Cr EDTA changed only slightly from control value (5–7 ...) during the histamine infusion period or when both histamine and isoprenaline infusions were performed. This is in agreement with recently published results from this laboratory. The PS values measured during the histamine infusion period seem to have

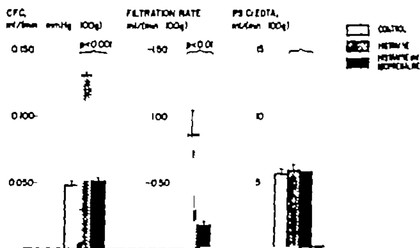


Fig. — CFC, filtration rate and PS for Cr EDTA (mean ± S.E.) in the maximally vasodilated hindpaw preparation at control, some 5–8 min after the start of the histamine infusion and around 10 min after the start of the combined histamine-isoprenaline infusion.

mainly reflected the diffusional exchange of Cr EDTA and only to a very limited extent conventional exchange. The reason is that histamine increases the albumin concentration in the filtrate to near plasma levels (Appelgren, Jacobson and Kjellmer 1966). Thus the non permeant tracer (C–G albumin) is filtered through the exchange vessels at almost the same rate as water. Consequently the increase in relative concentration of the non-permeant tracer in the venous affluent normally seen during filtration is very slight and the measured extraction fraction now greatly underestimates the bulk flow contribution to the solute

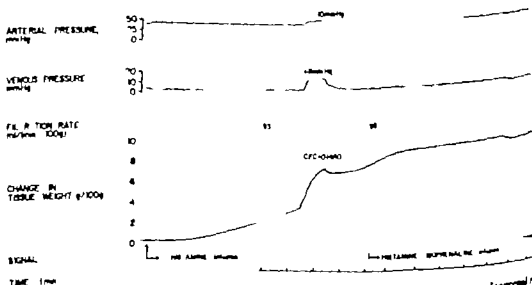


Fig. 3 The effect of isoprenaline on histamine induced changes in capillary fluid equilibrium. Isoprenaline changed the rate of filtration during histamine infusion from 0.91 ml/min 100 g of tissue to 0.11 ml and 100 g of tissue in 2 5 min. In this expt. control CFC was 0.413 ml/min mmHg 100 g while the CFC value during histamine is shown in the Figure. The CFC value measured 10 min after the start of the histamine-isoprenaline infusion was 0.0456 ml/min mmHg 100 g. Flow P_A and P_V were held constant throughout the recording with exception made for the period of CTC measurement.

rt. In the present histamine expts. this was calculated to at maximum 7–8% of total rt.

5 animals, which responded with only "moderate" changes in CFC (50–150% increase) comparably low filtration rates (0.25–0.50 ml/min 100 g) to histamine, and which excluded from the further investigation, since it was deemed desirable to have initially al histamine effects, were all perfused with fresh horse serum in dextran-Tyrode n. Full histamine responses could not be obtained unless bovine albumin or aged serum (stored for 3 weeks at 4°C) was used instead of fresh horse serum in the ate.

Action of histamine on the isoprenaline pretreated hindquarter vascular bed. It was a ent finding that pretreatment with isoprenaline rendered the hindquarter vascular argely insensitive to histamine, and this effect lasted for at least 60 min after stopping oprenaline infusion. Thus, no significant changes in fluid filtration CFC or in PS for DTA ere observed during the isoproterenol infusion or during the isoproterenol-mine infusion period.

Approximate dose levels of isoprenaline required to suppress histamine induced permeability ges. Complete inhibition of the histamine-induced changes in microvascular permea- as in 4 expts. obtained at isoprenaline concentrations of 40–70 ng/ml perfusate (mean 52 ng/ml), but partial suppression of the histamine effects was seen already at around ng/ml perfusate (~3–5 10^{-8} M) of isoprenaline. It should then be stressed that this the case despite the use of supramaximal histamine concentrations.

Action of histamine on the cooled hindquarter vascular bed. Control value of CFC be cooling was 0.0425 ± 0.0016 ml/min mmHg 100 g, which is somewhat lower than value given above. The difference is probably due to differences in perfusate, because he present group only aged horse serum was used as colloid in the dextran-Tyrode solu n and in the histamine-isoprenaline groups half of the expts. were performed with bovine umin in the perfusate. With the latter perfusate control CFC were always higher than h the former. At 10–15°C CFC decreased to 0.0281 ± 0.0010 ml/min mmHg 100 g 0.001). Peripheral vascular resistance increased from 2.98 ± 0.06 mmHg ml min 0 g to 4.51 0.11 mmHg ml min 100 g. The reaction of the cooled microvascular mbrane to histamine was delayed and markedly depressed. During the first 5–7 min of tamine infusion the cooled preparation was almost completely isogravimetric as shown Fig. 4 upper part but it then started to filtrate at low rate. CFC increased some 50–60% 0443 0.0164 ml/min mmHg 100 g (p 0.06). After stopping the histamine infusion d after rewarming CFC returned to control value. Histamine stimulation now produced n almost threefold increase in CFC and also marked filtration. The changes are summarized n Fig. 5.

Discussion

Histamine markedly increased CFC and caused net fluid filtration in the initially isogravi- metric rat hindquarter preparation. As the vasculature was already maximally dilated, evidenced by the failure of histamine to further change arterial and venous pressure or the PS for Cr-EDTA, the changes in CFC must be exclusively attributable to change in

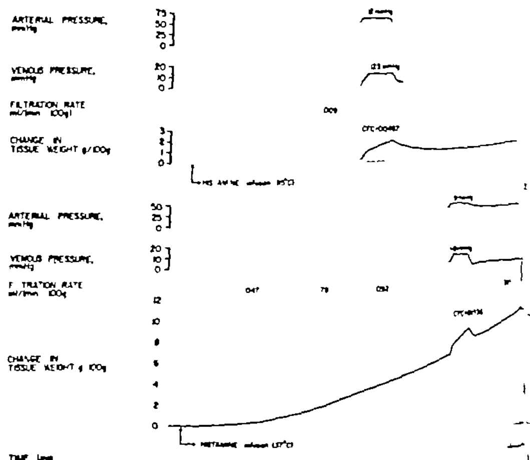
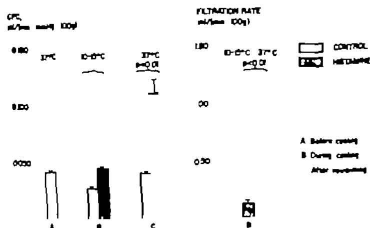


Fig. 4 CFC and capillary fluid equilibrium after histamine during cooling (upper part) and after rewarming (lower part) 30–40 min later in the same expt. The arterial pressure in this expt. was around 52 mmHg during cooling and around 37 mmHg after rewarming while flow was kept constant (1.2 ml/min 100 g) as well as the venous pressure (3 mmHg).

microvascular permeability rather than to any change in perfused capillary surface area. For essentially the same reasons the histamine-induced increase in net fluid filtration cannot be attributed to changes in microvascular hydrostatic pressure and surface area, but must reflect a decrease in the transmural colloid osmotic pressure difference, caused by an increased permeability to macromolecules. However despite these large changes in microvascular hydraulic conductivity and permeability to macromolecules, permeability to small molecules was not significantly altered from control during the histamine infusion. This is inferred from the PS values for Cr EDTA which failed to change significantly in response to histamine as more extensively dealt with in another study already published as a brief communication (Rippe, Kamiya and Folkow 1978). This discrepancy can best be explained by a preferential action of histamine on venular gap formation (large pores), resulting in an increase in their number and possibly their radius. The large pore to small pore ratio seems to be approximately 1 to 30 000 (Grotte 1956) up to 1 million (Winne 1965, Renkin 1970) of the total population of pores. By virtue of their big radius, 25 nm (Grotte 1956) up to ~160 nm (Renkin, Carter and Joyner 1974), the large pores are of particular importance for the movement of macromolecules from blood to interstitium and also for hydraulic



The effect of cooling and subsequent rewarming on CFC during control and during supramaximal ... infusion (mean ± S.E.) (left part). The maximal filtration rates during histamine infusion, during ... and during subsequent rewarming are also shown (right part).

...ductivity as this increases with the fourth power of the radius. However, as a result of ... small total cross sectional area relative to the small pore system the large pores exert less influence on the diffusion transfer of small molecules, which changes only with the ...nd power of the radius. For such reasons, small molecular permeability would be only ... affected even if the number or/and radius of large pores is markedly increased. For ...mple, if their number is increased 50 times CFC would theoretically increase 3 to 4 times ... the microvascular filtrate concentration of albumin about ten times (cf. Appelgren, ...obson and Kjellmer 1966), while the increase in effective pore area for small molecular ...fusion could be less than 10% calculated from Grotte's data (Grotte 1956).

The marked increase in CFC caused by histamine could be largely prevented by the ...ultaneous infusion of the catecholamine isoprenaline. Since all hemodynamic factors as ...ll as the diffusion capacity for small molecules were essentially unchanged relative to ...ntrol during the combined histamine-isoprenaline infusion, a decrease in microvascular ...rface area cannot have contributed to the failure of histamine to increase CFC under ...ese conditions. Instead, it is more likely that isoprenaline prevents the histamine-induced ...crease in microvascular permeability to macromolecules by a direct action on the micro-...scular membrane which counteracts that of histamine. This counteracting of histamine ...acromolecular efflux by isoprenaline is apparently a physiological antagonism and not a ...atter of histamine receptor blockade. It has been reported that histamine increases the ...umber of large pores in the immediate postcapillary venules by causing adjacent endothelial ...lls to "round up", thereby creating gaps presumably as a result of contraction of actomyo-...in-like anchoring filaments in the endothelial cells (cf. Haddy, Scott and Grega 1976). As ...he antagonism by isoprenaline does not involve histamine receptors, it is logical to assume ...hat isoprenaline via beta-adrenergic receptors causes relaxation of these filaments counter-...acting the contracting action by histamine. Svensjö, Persson and Arfors (1976) noted in a ...preliminary report that the marked increase in the number of venular leaks of FITC-dextran

(MW 150 000), caused by topically applied bradykinin, could be greatly reduced by the simultaneous topical application of the beta 2 adrenergic receptor agonist Terbutaline. The leaks are only found in the venules and represent gap formations between adjacent endothelial cells. As bradykinin acts similarly to histamine with regard to the site and magnitude of macromolecular efflux, it is reasonable to accept a similar hypothesis for the antagonism of histamine macromolecular efflux by catecholamines.

The great increase in CFC while PS for Cr EDTA is essentially unchanged upon histamine administration fails to support recent suggestions (Renkin, Carter and Joyner 1974; Carter, Joyner and Renkin 1974) that histamine should primarily enhance vesicular transport rather than increase the number of large pores, because in such a case changes in CFC could hardly be expected (*cf* Renkin 1977). Further, Rippe, Kamiya and Folkow (1978) demonstrated that marked cooling of the vasculature of the rat hindquarters via its perfusate failed to alter the clearance of macromolecules subsequent to increases in venous pressure relative to the situation in a parallel perfused preparation kept at 37°C. If transendothelial protein transfer really occurred by means of micropinocytosis, which at least in some of its links must represent energy dependent processes, the above findings would be impossible to explain while they are in precise agreement with macromolecular transfer via large pore filtration. In addition, as noted in this study severe cooling markedly reduced the effects of the increase in microvascular permeability to macromolecules and CFC by histamine, an effect that was immediately reversible by rewarming the perfusate to 37°C. When considered together, these data suggest that the microcontractile effect on venular endothelial cells by histamine is temperature sensitive and involves opening of intra-endothelial gaps other than the large pores responsible for the normal transfer of macromolecules. Furthermore, histamine can hardly exert these effects uniformly on the small pore system as this would invariably be reflected by changes in small molecular transfer by means of diffusion, which was not the case.

The effects of severe cooling on antagonizing the histamine actions on the venular membrane could explain the beneficial effects of "ice packs" in the treatment of various inflammatory and burn responses. It has been assumed that the marked reductions in tissue swelling by severe cooling simply reflected decreases in blood flow into the injured tissue. Perhaps an equally important action of severe cooling results from an antagonism of the microvascular actions of histamine and other mediators of inflammation by cold diminishing the development of venular gaps which would greatly reduce protein and fluid loss.

Additionally, the data in this study demonstrate that isoprenaline antagonizes the actions of histamine on macromolecular efflux even if given after the onset of the histamine infusion, at which time the effects of histamine are already maximal and remain so as long as the histamine infusion continues. Shortly after initiating the isoprenaline infusion, CFC returns to nearly control levels. It was also observed that after exposure of the rat hindquarters to isoprenaline, the microvascular membrane remained relatively refractory to histamine for 30 to 90 min despite the fact that isoprenaline was not infused during that time. Relative refractoriness to topically applied bradykinin for 30 to 90 min was also observed in the hamster cheek pouch after exposure to isoprenaline (Grega, to be published).

It is intriguing to speculate on the possible physiological significance of these unique

s of catecholamines on the microvascular membrane. While there is still no evidence ting that catecholamines are normally involved in the regulation of microvascular ability to macromolecules, this action of catecholamines to reduce permeability would paramount importance mainly in patho-physiological states associated with wide l and massive histamine and bradykinin release. These often shock-like states are y associated with profound hypotension which would function as a stimulus for an sed sympatho-adrenal discharge. Additionally high concentrations of histamine and kinin elicit a direct release of catecholamines from the adrenal medulla. The an-atic action of the catecholamines could prevent serious circulatory derangements from ve protein and fluid loss. In contrast, during *local* inflammatory responses, the actions the edemogenic agents would be unopposed. These data could explain, in part, several ming by perplexing observations. In contrast to local intraarterial infusions of hista-in the dog forelimb systemic infusions of histamine either intravenously (Grega *et al.*) or into the left ventricular chamber (Marciniak *et al.* 1977) fail to promote edema ation and, instead, produce sustained net extravascular fluid reabsorption in the canine limbs. This route-dependent differential action of histamine cannot largely be explained hemodynamic differences or differences in blood concentrations of histamine as a func of the route of administration (Marciniak *et al.* 1977 a), and, therefore, must represent agonism of the histamine action on the microvascular membrane. Further also in ated rat hindquarters perfused with horse serum, the ability of histamine to increase d filtration and CFC varies directly with the age of the horse serum. The effect of infusion histamine is reduced during perfusion of the rat hindquarters with fresh horse serum. In ntrast, histamine exerts marked effects on microvascular permeability to plasma proteins d CFC when perfused with aged horse serum (that which has been stored for 2–3 weeks). is tempting to speculate whether this discrepancy might be due to the possibility that esh horse serum contains liberated catecholamines, which may antagonize the actions of stamine on the microvascular membrane and that these factors might be slowly destroyed ring the period of storage. Additionally other biogenic agents present in the serum might so function as physiological antagonists of histamine and contribute significantly to these characteristic actions of histamine.

This study was supported by the Swedish Medical Research Council (14X-00016), by Svenska Sällskapet för Medicinsk Forskning, by the Medical Faculty, University of Göteborg and by National Institutes of Health research service award IF 32 HL 05409-01 from the National Heart, Lung and Blood Institute while Dr Grega was on sabbatical study at the University of Göteborg, Department of Physiology, from Michigan State University, East Lansing, Michigan.
The authors are indebted to Professor Björn Folkow for helpful criticism and suggestions throughout the course of this study. Thanks are due to Mrs Gertrud Karlsson for skilful and devoted technical assistance.

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Acta physiol. scand. 1978. 103. 263–269

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Aspects of temperature regulation in harp seal pups evaluated by *in vivo* experiments and computer simulations

By

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Received 12 December 1977

Abstract

…LAND, N. A. and K. RONALD *Aspects of temperature regulation in harp seal pups evaluated by* in vivo *experiments and computer simulations*. Acta physiol. scand. 1978. 103 263–269

…rature regulation in harp seal pups (*Pagophilus groenlandicus*) has been studied by *in vivo* measure… of oxygen consumption, body temperatures and *in vitro* determinations of the thermal values of …mple. The experimental animals were restrained and exposed to natural weather conditions. Solar …ance above 600 W/m (global radiation) caused hyperthermia. Computer simulations based on the …al values of the fur indicated that heat dissipation from primarily the limbs is a prerequisite for …ing hyperthermia under natural weather conditions. Further simulations showed that the lower …al temperature of a lean newborn harp seal pup with standard metabolism is only −1°C while it is …ased to 39°C as the pup grows, acquires a 10 cm thick layer of blubber and the metabolism …ses to 1.5 times standard. High metabolism and tolerance to low deep body temperatures (35°C) … prerequisites for thermal balance in the harp seal pup under cold conditions.

…words: Metabolism, body temperatures, wind speed, solar radiation, fur insulation, thermal …bution model

…he spring the harp seal (*Pagophilus groenlandicus*) hauls up on drifting ice to give birth …hite-furred pups. 2 or 4 weeks after birth the "whitecoats" grow from 7 to about 35 kg …nly by deposition of subcutaneous layers of blubber up to 10 cm thick. The white fur …ut 23 mm thick, is shed 2 to 4 weeks after birth (Sivertsen 1941 Frisch and Øritsland …).

…t birth the seal is exposed to and copes with, a variable and often harsh teh thermal …ironment The seal' thermoregulatory mechanisms have been investigated by only few …rkers Davydov and Makarova (1965) found that the heat production of the newborn … doubled from 73 to 145 W with immersion int ice water while there would be only …mall metabolic response to cooling at 2 to 4 weeks of age. A high metabolic response …cold air has been reported for moulting whitecoated harp seals (Iversen and Krog 1973).

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The thermal conductance of the fur has been reported to be 1.9 to 2.8 [illegible] in still air and increasing with 0.05 times the square power of the wind speed [illegible] Øritsland 1968, Øritsland *et al.* 1978). Grav *et al.* 1974 reported the presence of [illegible] high amounts of brown fat in the blubber layers of the newborn (7 to 14 kg) [illegible] Øritsland and Ronald (1973) found that solar radiation has a profound heating [illegible] on the skin of whitecoats lying on the ice and suggested that critical air temperatures [illegible] mined in the laboratory can not be used to predict metabolism and behaviour under [illegible] conditions.

Thus a good understanding of several aspects of the termal adaptations of the harp seal pups has been reached. However, an integrated quantitative description of heat [illegible] under natural weather conditions still is needed both for predictions of survival capacity of this species and because of the general need for heat balance models in ecological analysis.

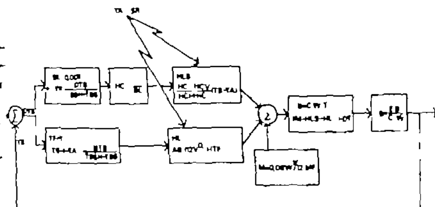
Towards producing such a model metabolism and body temperatures were measured [illegible] 7 whitecoated seals exposed to natural weather conditions and the effect of solar radiation on the heat transfer through a fur sample was determined. A thermal simulation model encompassing the conventional ways of describing metabolism, tissue and fur [illegible] body surface areas and dry weather conditions was programmed in APL (Iverson [illegible]). The model was used to evaluate the thermoregulatory capacity of the harp seal pup.

Materials and methods

Harp seal pups were transported in from the ice off the Magdalen Islands, Canada, 4 to 24 h prior to [illegible] periments. During measurement the experimental animal was secured to a plywood board by [illegible] aluminium hoops. A respiration valve was mounted close to the seal's nostrils and sealed off [illegible] respiration mask firmly covering the head. A Technology Versatronics OCC1000 continuous oxygen con-sumption computer was calibrated with a Kofranyi-Michaelis dry gas meter used in conjunction with [illegible] Scholander 0.5 ml analyzer (Cimolazio *et al.* 1963). The oxygen computer agreed within 5 [illegible] gasmeter—0.5 ml system. Temperatures were recorded with a thermistor probe inserted about 30 cm [illegible] the rectum and by a thermistor needle held parallel to the skin surface. Thermal properties of the [illegible] sample exposed to solar radiation were determined using a heat flow apparatus as described by Øritsland and Lavigne (1976). An Eppley solarimeter was placed level to the fur. Solar irradiance [illegible] alternately shading off and exposing the fur sample for a given set of air temperature and [illegible] through natural variations in solar irradiance.

A simulation model based mostly on conventional thermal physiological parameters was programmed in APL according to the control flow-diagram given in Fig. 1. The transfer functions used are [illegible] in the diagram, and the variables are as follows:

TB	= deep body temperature	°C
TBS	= set point and lower value of regulation band for deep body temperature	°C
TBSH	= upper value for regulation band	°C
DTB	= error signal (DTB = TB − TBS)	°C
TA	= air temperature	°C
V	= wind speed	$m s^{-1}$
SR	= solar irradiance (global)	$W m^{-2}$
HCI	= heat transfer coefficient peripheral tissues (HCI = KF/BL)	$W m^{-2} °C^{-1}$
KF	= heat conductivity of fat (0.205 See Carlson and Hsieh 1970)	$W m^{-1} °C^{-1} m^{-1}$
BL	= "functional" blubber thickness	m
AB	= body surface area excluding the flippers	m^2
HCV	= heat transfer coefficient of the fur (HCV = 1.9 + 0.05 V^2)	$W m^{-2} °C^{-1}$



1 Control flow diagram for simulation of heat balance of harp seal pups. Deep body temperature compared with the reference temperature (TBS) and the error signal (DTB) is used to determine functional blubber thickness (BL) and the flipper surface temperature (TF). Heat loss from the body (HLB) is based on the blubber and fur insulation being coupled in series while the heat loss from the flippers (HLS) is considered to parallel HLB. Heat production (M) is not coupled to the controller. The balance between heat loss and heat production determines the energy content (EB) of the body and thus deep body temperature. Disturbing elements are air temperature (TA), wind speed (V) and solar radiation (SR). The effect of solar radiation is to decrease the heat loss from the body (calculations are indicated in the figure) with a factor of 0.18 for 45% of the body surface excluding the flippers.

	flipper surface temperature	°C
	flipper surface area	m^2
	metabolism	W
S	heat loss from flippers	$W m^{-2}$
B	heat loss from body	$W m^{-2}$
	time between calculated values of TB	
	body weight	kg
	specific heat capacity of body	$J kg^{-1}\,°C^{-1}$
	energy (heat) content of body	J

*The simulation model is based on proportional control of blubber/skin heat transfer coefficient and flipper temperature and heat flows are calculated for plane surfaces and layers.

Results

Air temperatures ranged between −2 to 23°C, wind speeds 0.1 to 5 m/s and solar irradiance (global radiation) ranged up to 800 $W m^{-2}$ during the *in vivo* experiments. No significant metabolic response to changes in heat load was found. However deep body temperatures varied between 35°C and 41°C dependent on weather conditions. Low deep body temperatures were found in newborn (7–12 kg) pups both during experiments and when kept for about 12 h in an outside enclosure at air temperatures around 0°C, wind speed 2 $m s^{-1}$ and cloudy rainy and sleeting weather. High body temperatures were caused by solar irradiance above 600 $W m^{-2}$. Such solar irradiance gave skin temperatures from 38 to 41°C on the exposed side of the body. The metabolism was 1.5 ± 0.32 (S.D.) times higher than what could be expected from Kleiber's formula (Kleiber 1961). A summary of the experiments is presented in Table 1.

TABLE 1 Summary of measurements of metabolism in harp seal pups exposed to natural weather conditions. Metabolism were read with about 10 min intervals and values are calculated for U/h per l O_2 STP

Seal No	Weight kg	Mean metabolism W (S.D.)	Duration of experiment min	Windspeed ms^{-1}	Environmental conditions	
					Air temperature °C	Solar irradiance Wm^{-2}
1	8.3	24.5 ± 5.5	529	0–7	7 to 23	0
2	10.5	24.7 ± 1.9	220	1 2	1 to 2	20-422
3	10.5	24.6 ± 1.9	490	0.5–3	−2 to 2.5	<20
4	9	27.2 ± 3.3	493	4–5	−2 to 1	29-770
5	19	67.. ± 20.8	170	1 2	−1.5 to 1.5	30-100
6	20	49.0 ± 8.9	290	0–3	1.5 to 9	90-700
7	20	42.9 ± 7.8	257	0.5	−2 to 0	90-740

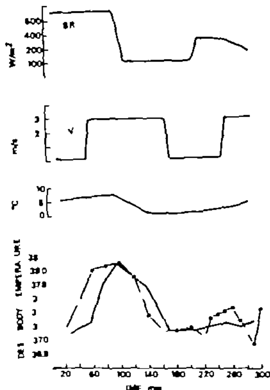
The ratio U also named the solar utilization factor is the heat load (at skin level) due to solar radiation divided by the solar irradiance (Øritsland 1971). In 8 experiments where solar irradiance was changed by alternate shadowing and exposure of the fur sample at constant wind speed and air temperatures, it was found to be 0.19 ± 0.09 (S.D.). For 5 expts the heat loss decrement due to solar radiation was calculated as the difference between expected and actual heat loss during continuous exposure of the fur sample. The expected heat loss was the heat transfer coefficient (Øritsland *et al.* 1977) multiplied by the skin to air temperature gradient. This resulted in a utilization factor (U) of 0.11 ± 0.08. Multiple regression analysis indicated that only 10 per cent of the variation in U was due to variations in wind speed and air temperature.

The average difference between simulated and *in vivo* deep body temperatures was less than 0.5 ± 0.7 (S.D.) °C for simulations of the separate expts. Such a simulation fidelity was obtained by using reference temperatures between 36.5 and 37.5°C and metabolism between 1.2 and 2 times standard (Kleiber).

Simulation of deep body temperature of a 20 kg whitecoat (seal no. 6) with specific heat capacity 3 100 kg °C and a constant metabolism 2 times what could be expected from Kleibers formula is presented together with *in vivo* values in Fig. 2.

Simulation of a small, lean whitecoat (10 kg and 1 cm of blubber) with standard metabolism resulted in a lower critical temperature (at wind speed 0.2 m/s) of −1 ± 1°C while with a blubber thickness of 10 cm the critical air temperature was −23 ± 1 °C. Simulating a large and lean (20 kg and 1 cm blubber) whitecoat having standard metabolism, resulted in critical air temperature of −3 ± 1 °C, while increasing the blubber thickness to 10 cm gave a lower critical air temperature of −27 ± 1 °C. For metabolism 1.5 times standard, the critical air temperature was −59 ± 1 °C for the 20 kg whitecoat with 10 cm blubber.

Also by simulation the whitecoats would rapidly become hyperthermic if the heat loss through the flippers was set to zero. For a 20 kg whitecoat the upper critical temperature for 1.5 times standard metabolism without heat loss through the flippers, was only −21 ± 1°C. Intact flippers allowed maintenance of constant deep body temperatures at air temperatures up to 15 ± 1 °C. Heat dissipation through flippers was also necessary for the simulated harp seal pup to survive solar radiation below 600 Wm^{-2}.



2 Simulated (●) and in rare ( ) deep temperatures of 28 kg haircoat ex d to varying levels of solar radiation wind speed (V) and air temperatures

Discussion

The present variability and range in deep body temperatures is wider than reported earlier (... cit.) while skin temperatures were in agreement to the values found on free whitecoats the ice (Øritsland and Ronald 1973). A similar variability in deep body temperatures has also been found by others working on unrestrained northern fur seal pups (Blix pers. comm.).

In a hooded seal weighing approximately 200 kg left to cool for 65 h *post mortem* the rectal cooling, measured about 50 cm from the tail, was 0.29 C/h, while in another central point close to the largest body diameter (about 1.2 m anterior to the tail) the cooling rate was 0.22°C/h at air temperature 8 to 1°C (Øritsland unpublished).

Thus a higher heat loss coefficient or surface to heat capacity ratio is present for the posterior as compared to the mid part of the body. Consequently the mid part can be expected to show more stable temperatures. Also, on the basis of the rapid shifts in blood circulation recorded in conjunction with diving reflexes (Blix 1976), resulting in cooling and heating of peripheral tissues, it is possible that the animals are able to adjust their insulation and deep body temperatures rapidly. Further studies encompassing simultaneous measurements of rectal and other locations for deep body temperatures are necessary in order to elucidate this hypothesis.

TABLE I Summary of measurements of metabolism in harp seal pups exposed to natural solar conditions. Metabolism were read with about 10 min intervals and values are calculated in W per l O_2 STP

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5	19	67.2 ± 20.8	170	1 2	−1.5 to 1.5	30-180
6	20	49.0 ± 8.9	290	0–3	1.5 to 9	90-700
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...d to direct sunshine, the pups seems incapable of reducing the metabolism sufficient ...ent overheating, and therefore depends on heat dissipation from primarily the ...h and to some extent the skin in general.

...eat balance model has been produced and can be used as demonstrated above in ...a of physiological responses to changing weather conditions. In addition the model ...obably be useful in future assessments of energy balance of other marine mammals.

...ted by the Canadian National Sportsmen Show WWF/Canada and the National Research ...l of Canada. Thanks to J. Krog and A. S. Blix for their comments to the manuscript.

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Significant variability in deep body temperature also has been noted in small [illegible] and birds and can partially be explained by changes in the reference or preferred body temperature (Morhardt 1975) Such changes are indicated by the present work as [illegible] varying from 36 5 to 37.5 were necessary to obtain acceptable simulations of the [illegible] expts.

The simulations employed a specific heat capacity of 3 100 Jkg $^{\circ}C^{-1}$ corresponding to a 0 23 to 0 77 ratio of fat to lean body mass with specific heat capacities of [illegible] 3 470 Jkg C respectively (Burton 1935 Minard 1970). Such a body composition [illegible] acceptable (Sivertsen 1941) and it thus appears that treating the body of the harp seal [illegible] as a composite of plane surfaces and layers related to the 2/3 power of the body [illegible] (Irving *et al* 1935 Iversen and Krog 1973) is valid in thermal simulations. While [illegible] experiments remain to be performed the present work and computer simulations reflect quantitatively the thermal significance of the blubber and supports the suggestion of Øritsland (1968) that there is a significant circulation through these tissues in response to heat stress.

Wide thermoregulatory variations in blood circulation in the flippers is suggested by the present work to act not only towards maintaining deep body temperature in the cold but for avoiding hyperthermia, the latter because of the limited heat loss that can be caused by circulation of the blubber Also the present work supports the suggestion of Øritsland and Ronald (1973) that critical air temperatures determined in the laboratory are not sufficient to describe mammalian heat balance under field conditions [illegible] appropriate heat load indices (*e g* Øritsland 1974) have been previously established.

The present metabolism values averaging 1.5 times standard are in close agreement [illegible] those reported earlier as reviewed by Irving (1972). In contrast to results published previously (Davydov and Makarova 1964 Iversen and Krog 1973) a metabolic response to cold was not found However the metabolic values reported by the above [illegible] occurred in response to more severe cooling (immersion in ice water Davydov and Makarova) than in the present experiments, or was obtained from moulting pups (Iversen and Krog) with presumably impaired insulation Actually Frisch and Øritsland (1968) predicted that a 70°C drop in air temperature may cause a doubling of metabolism in the harp seal pup if peripheral circulation is not reduced when the white fur is shed Inasmuch the variance of the metabolic values obtained here may obscure an eventual metabolic response, the question about whether or not there is a direct temperature related control of metabolism remains unsolved An arrangement with less restraint of the experimental animal seems necessary in future examinations of metabolic responses to cold

The simulations revealed, however that a high metabolic rate is a prerequisite for thermal homeostasis even for the largest and fattest whitecoat and that most natural changes in the thermal environment can be compensated for by metabolic adjustments well inside the range recorded here

In conclusion the thermal survival of the harp seal pup seems to be accomplished through a combination of high metabolism, most likely accomplished by non shivering thermogenesis in brown fat and muscle (Grav and Blix 1976) and tolerance to low body temperatures under cold conditions. Under warm environmental conditions, especially when



A typical experiment showing the dose-dependent increase in glycerol outflow from isolated fat cells stimulated with 0.1–10 µM noradrenaline (NA). 10 µM NA produced a smaller response than 1 µM in two out of three experiments.

Methods and materials

Epididymal fat was obtained from decapitated male Sprague-Dawley rats (Anticimex) weighing approximately 200 g. Isolated fat cells were prepared according to Rodbell (1964) using 2 mg/ml of crude bacterial collagenase (Worthington Biochem. C). After the final washing 1 ml aliquot of packed cells was transferred to a temperature-controlled plastic chamber containing 2.2.6 ml Krebs-Ringer phosphate buffer 7.4 with half the recommended calcium concentration, 5.5 mM glucose and 1 bovine serum albumin (Sigma, St. Louis). The floating fat cells were subsequently "perfused" (Allen et al. 1973 b) with this buffer at 37 C and a rate of 2 ml/min. Drugs were infused via a side-arm before the chamber or in the case of theophylline, dissolved in the perfusion buffer.

Lipolysis was measured as glycerol production (nmol ml cells^{-1} min^{-1}). Glycerol was measured by the method of Laurell and Tibbling (1966), but the $ZnSO_4$–$Ba(OH)_2$ precipitation step was eliminated to improve the sensitivity. Standard curves for glycerol in the perfusion buffer gave expected values despite this modification. Theophylline and adenosine deaminase (Boehringer, Mannheim), which was dialysed before use, did not influence the buffer blanks always included in the assay.

Results

There was a basal outflow of glycerol from the isolated perfused fat cells of 4.0 ± 0.5 nmol ml cells^{-1} min^{-1} (n 31). Lipolysis was stimulated in a dose-dependent manner by noradrenaline (Fig. 1) and theophylline (Fig. 2). The combination of noradrenaline and theophylline resulted in additive effects preferentially when submaximally effective concentrations of noradrenaline were used (Table I).

Adenosine deaminase (0.8–1 µg/ml) was an ineffective lipolytic agent in 3 expts. with perfused fat cells. Thus, adenosine deaminase increased glycerol outflow by only 1.1 ± 0.4 nmol ml cells^{-1} min whereas 1 µM noradrenaline in the same cells caused an increase of 23.8 ± 5.3 nmol ml cells min. The enzyme did not increase lipolysis in fat cells stimulated by a submaximally effective concentration of noradrenaline either. Adenosine inhibited lipolysis in perfused fat cells stimulated by noradrenaline or by theophylline. A typical experiment is shown in Fig. 3. The data summarized in Table II show that adenosine antagonizes theophylline-induced lipolysis more effectively than that induced by noradrenaline.

Discussion

The present experiments demonstrate antilipolytic effects of adenosine in micromolar concentrations in perfused fat cells stimulated by noradrenaline or theophylline. The failure

Acta physiol. scand. 1978. 103. 270–274

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Antilipolytic effect of adenosine in isolated perifused fat cells

By

PAUL HJEMDAHL and ALF SOLLEVI

Received 15 December 1977

Abstract

HJEMDAHL, P and A. SOLLEVI. *Antilipolytic effect of adenosine in isolated perifused fat cells.* Acta physiol scand 1978. *103.* 270–274

Adenosine markedly inhibits cyclic AMP accumulation in isolated fat cells, whereas inhibitory effects of adenosine on lipolysis have been difficult to demonstrate. The present study has been performed on isolated "perifused" fat cells where continuous monitoring of the lipolytic rate is possible and where modulating substances, such as adenosine, are not allowed to accumulate. Adenosine deaminase was ineffective as a lipolytic agent in perifused fat cells, suggesting no important background activity of adenosine in this system. Micromolar concentrations of adenosine inhibited lipolysis induced by noradrenaline (0.3 µM) and theophylline (1 mM). Theophylline was an effective lipolytic agent also in perifused fat cells suggesting that antagonism of adenosine is not the major mode of action of this drug on fat cells.

Adenosine, which is formed by the degradation of ATP, may be an important modulator of lipolysis in adipose tissue (*cf.* Schwabe *et al.* 1975). This proposition is based on the findings that adenosine is released from fat cells *in vitro* (Schwabe *et al.* 1973) and that adenosine and analogues of adenosine are potent inhibitors of cyclic AMP accumulation in isolated fat cells (Fain *et al.* 1972, Ebert and Schwabe 1973, Stock and Prilop 1974, Hjemdahl and Fredholm 1976). There are, however, conflicting results as to the effect of adenosine on lipolysis in isolated fat cells. Thus, Ebert and Schwabe (1973) found that adenosine inhibited lipolysis, whereas other studies (Fain *et al.* 1972, Fain 1973, Stock and Prilop 1974, Hjemdahl and Fredholm 1976) have failed to reveal an antilipolytic action of adenosine or adenosine analogues in concentrations producing virtually complete inhibition of the cyclic AMP response of the fat cells. This apparent lack of antilipolytic effect of adenosine might be due to a high background activity of adenosine in conventional fat cell incubates. We have therefore studied the effect of adenosine on lipolysis in "perifused" fat cells (Allen *et al.* 1973 b), a system which prevents accumulation of modulating substances such as adenosine in the vicinity of the fat cells. We have also used this experimental model to examine the effect of adenosine deaminase and theophylline, two drugs which may induce lipolysis by antagonism of adenosine (Schwabe and Evert 1974), in a situation where a low concentration of adenosine should prevail.

III The influence of adenosine (1 10 μM) on lipolysis in perfused fat cells stimulated by noradrenaline or by theophylline Note that theophylline-stimulated lipolysis is more sensitive to adenosine than noradrenaline-stimulated lipolysis

[illegible]ting agent	Net glycerol production rate (nmol min⁻¹ ml cells⁻¹)	% inhibition by adenosine 1 μM	10 μM
[illegible]M noradrenaline	22 ± 9	29 ± 6 (3)*	—
[illegible] noradrenaline	36 ± 12	25 ± 7 (− 5)*	34 ± 7 (3)
[illegible] theophylline	24 ± 5	59 ± 5 (4)*	78 ± 5 (4)*

[illegible]05, ** p 0.01 *** p 0.001

[illegible]ts of adenosine deaminase are due to removal of adenosine, since unspecific effects of [illegible] enzyme should have shown up also in perfused fat cells.

[illegible]he lipolytic effect of theophylline does not appear to be linked to its ability to inhibit [illegible]lic AMP breakdown by phosphodiesterase (Schwabe *et al* 1972, Allen *et al* 1973 a) [illegible]ead, it has been proposed that theophylline induces lipolysis by antagonizing effects of [illegible]acellular adenosine (Ebert and Schwabe 1973 Schwabe and Ebert 1974). This suggestion [illegible]ased on findings that theophylline inhibits effects of adenosine in many organs and that [illegible]ophylline and adenosine deaminase produce similar effects in conventionally incubated [illegible] cells.

The present experiments show that adenosine is a more potent antagonist of lipolysis [illegible]uced by theophylline than of that induced by noradrenaline. The potency of adenosine [illegible]y however be inversely related to the cyclic AMP levels of the fat cells (Fain 1973 [illegible]holm 1977). Therefore, the greater sensitivity of theophylline-induced lipolysis could [illegible] explained by the fact that theophylline increases cyclic AMP considerably less than does [illegible]radrenaline The dissociation between the effects of theophylline and adenosine deaminase [illegible]served in the present expts. with perfused fat cells is not easily reconciled with the above [illegible]ntioned idea that the major mode of action of theophylline is through antagonism of [illegible]enosine. The present findings do not rule out the possibility that adenosine and theo[illegible]ylline exert their effects on fat cells through a common mechanism, although they em[illegible]asize the need for further proof

[illegible]is study was supported by grants from Karolinska Institutet and the Swedish Medical Research Council [illegible] no 04X 2553)

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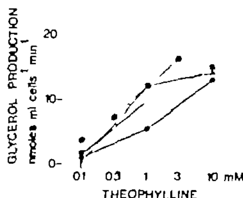


Fig. 2

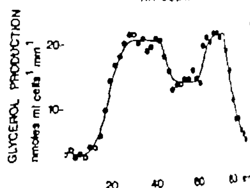


Fig. 3

Fig. 2. Dose-dependent glycerol production rate in four experiments with perifused fat cells stimulated by 0.1–10 mM theophylline. The experimental design was the same as that illustrated in Fig 1

Fig. 3 A typical experiment demonstrating the antilipolytic effect of 1 μM adenosine in perifused fat cells stimulated by 0.3 μM noradrenaline (NA).

in several previous studies to demonstrate antilipolytic effects of adenosine in concentrations which inhibit cyclic AMP accumulation in the fat cells markedly (Fain et al. 1972, Fain 1973, Stock and Prilop 1974, Hjemdahl and Fredholm 1976) is probably due to accumulation of adenosine in the incubates to such an extent that maximal antilipolytic effects of adenosine already prevailed

The technique of "perifusing" isolated fat cells (Allen et al 1973 b) was chosen for the present study because of the possibility of studying the effects of adenosine without the interference of a high background activity of adenosine, which accumulates rapidly in conventional fat cell incubates (Schwabe et al 1973). The failure to demonstrate lipolytic effects in perifused fat cells of adenosine deaminase, which induces lipolysis in conventionally incubated fat cells (Fain 1973, Schwabe and Ebert 1974, Hjemdahl and Fredholm 1976) may be taken as evidence that the perifusion technique reduces the adenosine concentration at the fat cell to unimportant levels. These results also support the contention that the

Table 1 The effect of theophylline (Theo) on lipolysis in perifused fat cells stimulated by noradrenaline (NA, 0.1–3 μM) in 6 separate expts. Note that theophylline caused further increase in glycerol production rate when submaximally effective concentrations of noradrenaline were used

NA (μM)	Theo (mM)	Glycerol production rate (nmol ml cells⁻¹ min⁻¹) during NA	NA + Theo	
0.1	0.1	46.4	72.5	(+56 %)
0.3	0.3	6.2	41.6	(+59 %)
1.0	1.0	18.8	[illegible]3.1	(+[illegible]3 %)
1.0	1.0	56.7	71.5	(+26 %)
1.0	3.0	[illegible]9.8	27.8	(− 7 %)
3.0	3.0	40.6	46.3	(+14 %)

Acta physiol. scand. 1978. 103. 275–283

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ra-abdominal pressure changes during natural movements in man

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Received 27 December 1977

Abstract

rillner, J. Nilsson and A. Thorstensson. *Intra-abdominal pressure changes during natural movements in man*. Acta physiol. scand. 1978. *103* 275–283.

weight of the upper part of the trunk is partially transmitted to the pelvis in the vertebral column muscle walls around the abdominal cavity are contracted, high pressure can be generated within the y (200 mmHg) The abdominal space can then transmit part of the weight to, e.g. the upper part he body Intra-abdominal pressure recordings have been performed during locomotion and other ral movements ith intragastric pressure recordings. With each step, there is phasic variation in sure, ith its peak coinciding ith that of the peak vertical force exerted by the leg gainst the nd. The peak values increase progressively ith the speed of alking/running up to a mean of mmHg and ith trough alues of 16 mmHg The phasic ariations ith each step is due to phasic vation of the abdominal muscles, ith an EMG activity starting 50 ms or more before foot contact. If ra load put on the back, the posture changes and at the highest speed of running the pressure es are significantly higher than without this additional load. After a jump down from moderate height 4 m, the average increase is 89 mmHg and can often exceed 100 mmHg. These pressure changes are ge and ill presumably act to unload the spine under the prevailing biomechanical conditions and, in tion, there will no doubt be an effect on the circulatory system.

rprisingly little detailed information is available on how the trunk is controlled during fferent types of movements, despite the fact that it represents the larger part of the ody weight, and consequently is of paramount importance in the equilibrium control uring locomotion and posture. One special aspect relates to the control of the pressure ithin the abdominal cavity

The load of the upper part of the body in man would entirely be transmitted to the elvic girdle by the ertebral column if the intraabdominal pressure (IAP) was zero. How er if a pressure is created in the abdominal ca ity by the joint action of the muscles of he pelvic floor the abdominal wall, and the diaphragm, this pressure can act t support he upper part of the body and thereby unload the spine in a degree proportional to the actual pressure (e.g. Bartelink 1957 Morris *et al.* 1961 Eie and Wehn 1962, Davis and Troup 1964). Indeed, pressure changes bove 200 mm Hg have been recorded during lifting (Morris *et al.* 1961 Eie and Wehn 1962). The degree of increase in pressure is related to the

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Acta physiol. scand. 1978, 103, 275–283

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,ra-abdominal pressure changes during natural movements in man

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Abstract

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eight of the upper part of the trunk is partially transmitted to the pelvis via the vertebral column. muscle walls around the abdominal cavity are contracted, high pressure can be generated within the (1 200 mmHg). The abdominal space can then transmit part of the weight to, e.g. the upper part of the body. Intra-abdominal pressure recordings have been performed during locomotion and other movements with intragastric pressure recordings. With each step there is phasic variation in pressure, with its peak coinciding with that of the peak vertical force exerted by the leg against the ground. The peak values increase progressively with the speed of walking/running up to mean of mmHg and with trough values of 16 mmHg. The phasic variations with each step is due to phasic action of the abdominal muscles, with an EMG activity starting 50 ms or more before foot contact. If extra load is put on the back, the posture changes and at the highest speed of running the pressure values are significantly higher than without the additional load. After jump down from moderate height 3.4 m, the average increase is 89 mmHg and can often exceed 100 mmHg. These pressure changes are large and will presumably act to unload the spine under the prevailing biomechanical conditions and, in addition, there will no doubt be an effect on the circulatory system.

Surprisingly little detailed information is available on how the trunk is controlled during different types of movements, despite the fact that it represents the larger part of the body weight, and consequently is of paramount importance in the equilibrium control during locomotion and posture. One special aspect relates to the control of the pressure within the abdominal cavity.

The load of the upper part of the body in man would entirely be transmitted to the pelvic girdle by the vertebral column if the intraabdominal pressure (IAP) was zero. However, if a pressure is created in the abdominal cavity by the joint action of the muscles of the pelvic floor, the abdominal wall, and the diaphragm, this pressure can act to support the upper part of the body and thereby unload the spine in a degree proportional to the actual pressure (e.g. Bartelink 1957, Morris *et al.* 1961, Eie and Wehn 1962, Davis and Troup 1964). Indeed, pressure changes above 100 mm Hg have been recorded during lifting (Morris *et al.* 1961, Eie and Wehn 1962). The degree of increase in pressure is related to the

weight lifted and to the horizontal distance from the load to the spine (Davis and Troup 1964 Asmussen and Poulsen 1968 Andersson *et al* 1976). These pressure increases primarily depending on active contractions of the oblique and the transverse abdominal muscles rather than the rectus abdominis (Bartelink 1957 Morris *et al* 1961). The degree of unloading of the spine is not clear but has been estimated to be 30-40% when lifting heavy weights (Morris *et al* 1961 Eie and Wehn 1962).

Except for Eie's (1966) data on skiing (ski-jumping (!)), nothing is known about the control of the intraabdominal pressure during normal activities such as walking, running, walking with a back pack or jumping down a few tenths of a meter In the present paper we have studied such natural motor activities. The specific questions we wanted to explore were

1 What changes occur in IAP during locomotion?
2. Does an extra load on the back influence the magnitude of the IAP during locomotion?
3 How are the changes in IAP related to the vertical force produced in each step or jump?
4 Are the changes in IAP related to active contractions of the muscles of the abdominal wall?
5 How is the EMG activity in the abdominal muscles related in time with the increase in IAP and the rise in vertical force during locomotion and jumping (i.e. is the development of IAP a pre programmed process)?

Some of these results have been reported in preliminary form (Thorstensson et al. 1977).

Material Methods and Procedure

Subjects

A total of 13 subjects, 1 males and one female, participated in the study Their mean age, height and weight were 25 yrs (range 18 34 yrs), 180 cm (range 173–187 cm) and 74 kg (range 65.5–87.0 kg), respectively All were "normal" subjects, habitually active but not involved in regular training None of the subjects had a history of back pai

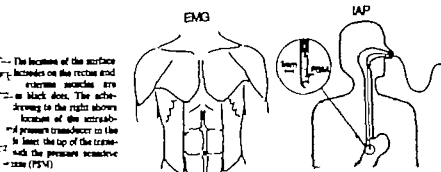
Methods

The intraabdominal pressure (IAP) was measured intragastrically with a tip pressure transducer (Millar Micro-tip, PC 340 Millar Instr Houston, Texas, USA) designed for pressure recordings intravascularly. The transducer had a frequency response DC to 20 kHz and a normal accuracy of within 0.5% of the selected range from −300 to +400 mmHg. Compared to the balloon that has often been used before for IAP recordings (e.g. Bartelink 1957 Morris *et al* 1961 Davis and Troup 1964 Eie and Wehn 1962 Asmussen and Poulsen 1968), the use of a transducer placed in the ventricle allows recordings of rapid pressure changes without any associated damping effects.

The transducer catheter was placed in flexible plastic tube (⌀ = 3.5 mm) and introduced through the nose after light local anaesthesia (Fig 1) The position of the transducer in the ventricle was easily verified by the behaviour of the pressure during deep breathing and Valsalva manoeuvres. In the ventricle the pressure sensitive membrane placed on the side of the catheter was allowed to protrude 2 3 mm from the end of the plastic tube (Fig. 1). Usually the distance from the nostrils to the transducer was 45-55 cm depending on the height of the subjects

The control unit (Millar TCB-100, weight 0.6 kg) was carried by the subject in a small case over the sternum. Electrical calibration was built into the control unit.

Control experiments were carried out with the transducer in a plastic or glass bottle carried by the subjects when performing the different exercises. The content of the bottles was varied to simulate in vivo conditions. In no case did the pressure under these artificial conditions exceed 8 mmHg.



... The location of the surface electrodes on the rectus and externus muscles are ... as black dots. The ... drawing to the right shows location of the intraabdominal pressure transducer in the ... In inset the tip of the transducer with the pressure sensitive membrane (PSM)

electromyographic (EMG) activity of the abdominal muscles was recorded with surface electrodes bilaterally on the rectus abdominis and the obliquus externus muscles according to Fig. 1. The ... between the electrodes was 4 cm. The frequency range was 30 Hz to 10 kHz. The preamplifiers ... P 15; total weight 2.2 kg) were attached to a specially made light-weight backpack frame.

... series of experiments were performed including walking and running either on a motor-driven treadmill (Series A), or over a force platform (Series B). Also running in place and different jumps were ... in Series B.

Series A. (... 10). Before the experiments on the treadmill started the subjects got used to the test ... in 2-3 training sessions. On the test day walking and running were performed at the belt speeds ..., and 12 km h⁻¹, running also at 15, 18 and 21 km h⁻¹. The velocities were applied in the following ... 6, 12, 9, 18, 15 and 21 km h⁻¹. A minimum of 20 consecutive steps of steady locomotion was ... at each velocity. Recovery periods of 1-2 min were allowed between each run. After a longer rest ... (5-10 min) the protocol was repeated with an extra back load. The female and 3 males weighing less ... 70 kg carried 19 kg load and the remainder carried 27 kg. The weight consisted of 5 iron bars 35 ... that were symmetrically placed close to the back on the frame mentioned above.

... mean IAP as well as the peak and the trough (min. IAP) values were recorded and related to the ... of locomotion and to the step sequence. The different phases of the step cycle were assessed by ... electrogoniometers placed over the knee joints, and in some expts. by microswitches mounted in the sole of ... conventional sport shoes used as test shoes.

Series B. (n ... 6). In this series different motor behaviours were performed on a force platform (Type ... A, Kistler Instr. AG, Winterthur, Switzerland), mounted level with the floor. The movement of the ... itself when loaded was negligible. The platform allowed measurements of 3 perpendicular force ... components. In the present study however only vertical force (the predominating force component) was ... together with IAP and EMG.

The speed of walking and running over the force platform was adjusted to correspond to approximately ... and 9 km h⁻¹ respectively. The speed of locomotion was assessed by measuring the duration of the foot ... contact and compare it with that obtained when walking and running on the treadmill with different velocities. The frequency of running and jumping in place was determined by a metronome. The jumps down ... to the platform were performed from a 40 cm high platform. At least 10 steps or jumps were recorded ... each experimental situation.

The IAP and the EMG were recorded simultaneously with the signals from the electrogoniometers and ... microswitches (Series A) or the vertical force (Series B) on an 8 channel recorder (Mingograf 803, Elema, Stockholm, Sweden), with a straight frequency response from 0 to 1 200 Hz.

Results

Walking and running

IAP in relation to speed. The IAP recordings during walking and running showed phasic changes. High and low pressures alternated in a regular fashion. The peak IAP demonstrated a steep, almost linear increase with increased speed both for walking and running

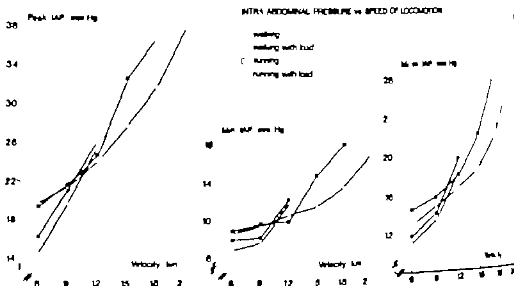


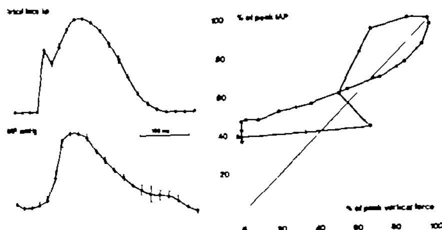
Fig. 2. The intraabdominal pressure (IAP) in relation to the speed of walking and running on a treadmill. The peak and the trough (min. IAP) values of the phasic IAP changes are shown together with the mean IAP calculated from the area under the pressure curve. The filled symbols represent IAP during walking and running with an extra back load of 18–7 kg. Values are means for 10 subjects and of 10 consecutive steps for each subject. The standard deviations, for practical reasons not included in the figure, range 3–7 mmHg.

(Fig. 2 left) reaching values around 38 mmHg at the highest running velocity. Also the min IAP and consequently the mean IAP values, increased with higher speeds of locomotion (Fig. 2 middle and right). The increase in min IAP was, however, not as steep as for peak IAP which resulted in an increase in the peak to peak values with speed, from about 8 mmHg at slow walking to 22 mmHg at the highest speed of running. It is noteworthy that during fast running a relatively high IAP is also maintained between the peak values. At the lowest velocity the peak IAP was higher for running than for walking. This difference was not present when walking and running were compared at higher velocities (Fig. 2).

The effect of an extra back load on IAP. The effect on the IAP of the symmetrically placed extra back load is shown in Fig. 2. The extra load caused a significant ($p < 0.05$) increase in IAP only at the highest speeds used (15 and 18 km h^{-1}). The behaviour of the IAP in relation to speed of locomotion was otherwise similar with and without load. The fastest speed (21 km h^{-1}) was not compared due to difficulties encountered by the subjects when running with the extra load at this velocity.

IAP in relation to vertical force in each step. Fig. 3 and 4 show the high degree of covariation seen in the development of IAP and vertical force produced in each running step. The IAP tended to build up somewhat before the instance of foot contact (Fig. 3 left) and was actually leading the development of vertical force except for the initial sharp rise in force (*cf.* Fig. 3 right).

In walking the situation was more complicated. During the support phase of one leg on the force platform both the vertical force and the IAP showed a biphasic increase. However, due to the occurrence of a double support phase during walking, it was difficult to exactly relate the increase in IAP to either the heel strike of one leg or the push-off of the other



3 The intraabdominal pressure in relation to vertical force exerted in one step when running over a platform with speed of approximately 9 km h^{-1}. Values are means $\pm$ 1 S.E. for 6 subjects each running 10 runs. To the right the two variables are compared in relative terms. The thin line ... y

EMG activity in the abdominal muscles. Having established that the changes in IAP re phasic and related to the step cycle it was of interest to see if the increase in IAP was a ssive phenomenon or if it was associated with phasic contractions of the abdominal uscles. Fig. 4 gives a typical example of EMG activity in the abdominal muscles during nning, this time performed in place on the platform to obtain force recordings from nsecutive steps. In each step there was a phasic activation of both the rectus abdominis d the oblique muscles. The EMG of both muscles started prior to foot contact and also fore the increase in IAP (Fig. 4). Thus it can be concluded that in each step there is a hasic activation of the abdominal muscles. This contraction creates the conditions necessary r the rapid increase in pressure (see discussion). No attempt was made to compare the EMG activity in the rectus abdominis and the oblique muscles on a quantitative basis. However the EMG of the oblique muscles appeared to be more consistently associated in line with the increase in IAP than the EMG of the rectus abdominis. The lateral part of the abdominal wall consists of three layers of muscles (the external oblique, the internal oblique



Fig. 4 Vertical force, intraabdominal pressure and EMG of the right rectus abdominis and oblique muscles during three consecutive steps in one subject running in place on force platform.

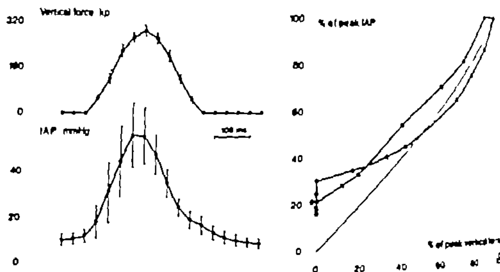


Fig. 5. The intraabdominal pressure in relation to vertical force exerted when jumping in place on a force platform. Values are means ± 1 S.E. for 6 subjects each performing 10 jumps. To the right the two variables are compared in relative terms (cf. Fig. 3).

and the transverse muscle). The cutaneous recording electrodes will of course pick up the largest signals from the external oblique muscle but will in all likelihood also pick up some activity from the deeper layers. However, it is likely that these muscles are usually coactivated since it would be desirable to have muscle fibres with different orientations activated at the same time.

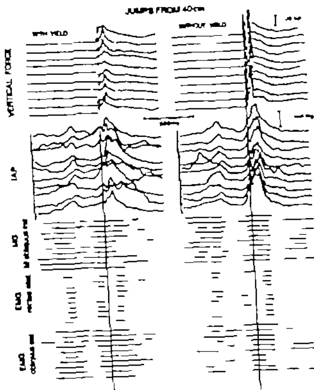
Jumping

Jumping in place. Different jumping regimes were performed on the force platform using both one and two legs. A good correlation was always seen between the IAP and the vertical force as shown for two leg jumps in Fig. 5. The covariation between IAP and vertical force was similar also to that seen in running (cf. Fig. 3). Compared to running the differences from one individual to the other were somewhat larger in jumping (Fig. 5).

Jumping down from a height. The jumps down onto the force platform from a height of 40 cm were done in two different ways, either with a yield in the hip, knee and ankle joints or without yield, i.e. hitting the platform with the instruction to keep the joints stiff. The jumps without yield caused the largest peaks in vertical force and IAP (Fig. 6). Peak IAP values exceeding 100 mmHg were often seen (the mean value for the 6 subjects was 89 ± 15 (S.E.) mmHg). The IAP started building up clearly before the instance of foot contact. The increase in IAP was associated with an increase in EMG activity in the abdominal muscles. Again the EMG activity of the oblique muscles was more consistently related to the rise in IAP than that of the rectus abdominis (Fig. 6). The EMG pattern appeared similar in jumping both with and without yield.

Discussion

The results of the present study have demonstrated substantial increases in pressure within the intra-abdominal cavity during common motor activities such as walking,



4. Superimposed recordings ...al force and intraabdominal pressure (IAP) in one subject ...orming jumps from 40 cm onto force platform, with ...(left) and without yield (right) ...he hip, knee and ankle joints. ...vertical lines denote the ... of foot-contact. The duration of the EMG activity in the ... abdominus and obliquus (bilaterally) muscles is shown below horizontal lines. The first peak of IAP and the corresponding EMG activity coincide with the ...off from the 40 cm plateau

...ning and jumping. The increases in IAP were phasic and occurred when the vertical load ...the body was increased. The rise in IAP was associated with increased EMG activity in ...e muscles of the abdominal wall. It is important to note that the phasic muscle activity ...started prior to foot contact. It forms clearly a part of the neural control system for ...comotion and is integrated into the "locomotor program" (see Grillner 1975).

It is striking how similar the changes in vertical force were to the intra-abdominal ...essure recordings (see Fig. 4). This is presumably related to two different things:

1 that (a) the relevant muscles are activated before and during each contact period (Fig. 4, 6) and (b) that the higher the level of pressure the larger the degree of muscle activity. It is noteworthy however that the degree of EMG activity was not following the pressure recordings closely (see Fig. 4).

... that actively contracting muscles produce more tension when extended (e.g. Rack and Westbury 1969). This increase in tension (stiffness in e.g. N mm⁻¹) will inevitably play a role during load compensation (e.g. Grillner 1972). The abdominal muscles form the wall of an elongated cavity similar to an elongated ball. In each contact period, the upper part of the body will bounce down and exert an extra pressure on this "ball". Depending on the stiffness of the muscle wall, a large or a small pressure will result within the cavity. The amount of pressure depends on the stiffness, which is related to the degree of muscle contraction. Although the stiffness of the muscles lining the abdominal cavity is unknown, it is clear

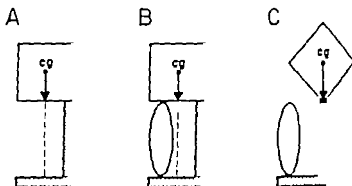


Fig. 7 Schematical representation of how the weight of the upper part of the body may be transmitted to the pelvis. In A only the vertebral column is assumed to contribute. In B the abdominal cavity is taking part of the weight. In C the vertebral column is again solely responsible, although an intra-abdominal pressure is built up. cg, abbreviation for center of gravity of the upper part of the body.

that this factor ought to contribute to the building up of pressure. These properties in themselves can then account for the similarity between the IAP and the vertical force.

The rigid structure (cylinder) thus formed by the contracting muscles of the abdominal wall allows a fast rise in IAP in response to sudden increases in vertical force, as e.g. in fast running or jumping down from a height. Such a mechanism obviates the time delays due to reflexes and electromechanical coupling (Grillner 1972). The latter factor being not least important in the present study, considering the rather sluggish system with the wide sheets of oblique and transverse abdominal muscles. This may also explain the often rather long (up to 50–100 ms) time lag between onset of EMG and rise of IAP.

It is beyond the scope of this article to estimate the relative importance of the abdominal "shock absorber" in relation to the vertebral column with its intervertebral discs. Clearly the importance will depend on a number of factors, some of which can be defined such as the transverse area of the abdominal cavity, the form of the cavity, the actual IAP, the inclination of the body etc. The last factor relates to the location of the mechanical axis. If the mechanical axis goes through the spine, the abdominal cavity will cause little unloading (Fig. 7 C). If, however, the mechanical axis is ventral to the spine, the abdominal cavity can taken up more pressure (Fig. 7 B). The amount of load that can be taken up depends on the stiffness of the walls, which is related to the degree of muscle contraction (see above). The actual load taken up may thus be either high or low when the IAP itself is high (compare Fig. 7 B and C). From this follows that a contraction of the abdominal muscles creating a high IAP (e.g. a Valsalva manoeuvre) does not automatically create an unloading effect on the spine. This may explain why Andersson *et al.* (1976) did not find a decreased disc pressure when Valsalva manoeuvres were induced. In their expts. the course of the mechanical axis is unknown — a further complication my be related to the fact that the pattern of muscle activity inducing a Valsalva (similar to defecation) differs substantially from that occurring *e.g.* during lifting.

It is noteworthy that an insufficiency in abdominal muscle function has been suggested as one possible factor in the etiology of back pain (*e.g.* Nachemson 1971). Everything else being equal, patients unable to build up adequate IAP would load their spine more

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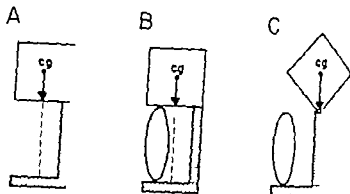


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Acta physiol scand. 1978. 103. 284–298

From the Laboratoire de Neurophysiologie, Collège de France, Paris, France

"Fast" and "slow" skeleto-fusimotor innervation in cat tenuissimus spindles, a study with the glycogen-depletion method

By

L. Jami, D. Lan-Couton, K. Malmgren and J. Petit

Received 30 December 1977

Abstract

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The glycogen-depletion method was used to investigat the mot r supply to tenuissimus with respect to the presence f fast β axons and to assess the total proportion of both fast and slow β-innervated spindles in this muscle In f rst series of 5 pts., groups f motor axons with conduction velocities higher than 85 m/s were repetil ely stimulated so as t produce glycogen depletion in the muscle fibres they innervated. The whole muscle was then quick-frozen, serially cut stained t demonstrate glycogen and examined for intrafusal glycogen depletion. Zones f glycogen depletion were found in 16 of the 46 examined spindles, they were most frequently located in the lo gest of the chain intrafusal muscle fibres. Since it is known that there are o purely fusimotor axon to tenuissimus ith conduction velocities above 50 m/s, it is concluded that β axons are present mo g the fastest ax n to this muscle In second series of 5 expts as many motor axons as possible with conduction eloc ties above 60 m/s were stimulated. Zones of glycogen depletion were fo nd in 19 of the 47 xamined pindles. They affected chain f bres in about half of the instances and bag f bres in the others. As this latter location is characteristic of slow dynamic β axons, it was concluded that both slow and fast β ax occur regularly in the motor supply to tenuissimus. β-I nervation is present in at least 40% of tenuissimus spindles with almost no convergence of fast and slow β axons onto the same spindle

Key ords: skeleto-fusimoto innervation β ons

Recent evidence indicate that there are two types of skeleto-fusimotor axons, also called β axons. The first and best known type of β axon was originally detected by physiological investigations (Bessou, Emonet Dénand and Laporte 1963 1965) and documented shortly after by histological findings (Adal and Barker 1965) Most of these β axons have conduction velocities in the 50–85 m/s range and on stimulation increase the dynamic sensitivity of spindle primary endings (Bessou *et al* 1965 Emonet Dénand, Jami and Laporte 1975, Mc William 1975). In accordance with these physiological characteristics, slow dynamic

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axons were found to innervate intrafusal muscle fibres of the bag type and extrafusal muscle fibres of the slow-oxidative type (Barker *et al.* 1977). Several studies have shown that this "slow" β innervation exists in various muscles of different species (see the review of Emonet-Dénand, Jami and Laporte 1978).

Much less is known about the second type of β axon. Histophysiological evidence for their existence (Harker *et al.* 1977) was first obtained from a study with the glycogen-depletion method of Edström and Kugelberg (1968). This study was carried out on the cat peroneus tertius muscle to investigate whether some motor axons in the 85–110 m/s conduction velocity range give collaterals to muscle spindles. Groups of motor axons with conduction velocities above 85 m/s were repetitively stimulated in order to produce glycogen depletion in the muscle fibres they innervated. The whole muscle was then quick-frozen, serially cut, stained to demonstrate glycogen and examined for intrafusal glycogen depletion. Zones of glycogen depletion were found in 27% of the examined spindles, they were almost exclusively located in the longest of the chain intrafusal muscle fibres. As it was further checked that there are no purely fusimotor fast axons to peroneus tertius (see also Emonet *et al.* 1972), the intrafusal glycogen depletion provided evidence that some of the motor axons to peroneus tertius in the 85–110 m/s range have a skeleto-fusimotor distribution, *i.e.*, they are β axons. The action of such "fast" β axons on spindle sensory endings is unknown but it can be expected that they are static since they innervate chain fibres.

In the present experiments, similar use was made of the glycogen-depletion method in order to ascertain whether "fast" and "slow" β innervation may coexist in the same muscle. It was first examined whether the cat tenuissimus muscle, already known to receive "slow" β innervation (Barker *et al.* 1971; Emonet-Dénand *et al.* 1975; Mc William 1975; Barker *et al.* 1977; Boyd *et al.* 1977) is also supplied by "fast" β axons. As this proved to be the case, an attempt was made to assess the overall incidence of both "slow" and "fast" skeleto-fusimotor innervation in tenuissimus spindles.

The two types of skeleto-fusimotor axons were termed "fast" and "slow" only on account of their conduction velocities. The fact that each type of β axon innervates a different type of intrafusal muscle fibre further suggests that they serve different functions, which appears to justify their distinction on the basis of conduction velocity. But it should be kept in mind that the limit of 85 m/s between "fast" and "slow" β axons was set empirically so that some overlap is to be expected. In the present study the term "slow" β axon will be used as a synonym of dynamic β axon supplying bag_1 intrafusal muscle fibres, whereas the term "fast" β axon will designate a β axon of unknown function supplying chain intrafusal muscle fibres.

Methods

Two series of 5 expts. each were carried out on adult cats anaesthetized with pentobarbital (Nembutal, Abbott Laboratories, 40 mg/kg I.P.). The first series was aimed at the detection of fast β axons innervating the tenuissimus and in each experiment of this series as many motor axons as possible with conduction velocities above 85 m/s were stimulated simultaneously (see Fig. 1 and Table I). The aim of the second series of experiments was to determine the total number of tenuissimus spindles innervated by β axons whether slow or fast. This implied the simultaneous stimulation of all motor axons to tenuissimus with the sole exception of γ axons; in practice, as many motor axons as possible with conduction velocities above 50 m/s were selected for stimulation in each experiment of this series (see Fig. 4 and Table II).

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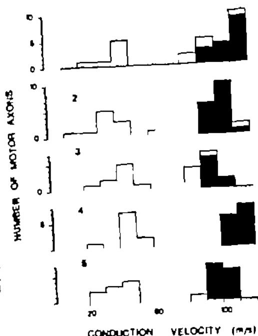
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The glycogen-depletion method was used to investigate the motor supply to tenuissimus with respect to the presence of fast β axons and to assess the total proportion of both fast and slow β-innervated spindles in this muscle In a first series of 5 expts., groups of motor axons with conduction velocities higher than 85 m/s were repetitively stimulated so as to produce glycogen depletion in the muscle fibres they innervated The whole muscle was then quick-frozen, serially cut, stained to demonstrate glycogen and examined for intrafusal glycogen depletion Zones of glycogen depletion were found in 16 of the 46 examined spindles, they were most frequently located in the longest of the chain intrafusal muscle fibres. Since it is known that there are no purely fusimotor axons to tenuissimus with conduction velocities above 50 m/s, it was concluded that β axons are present among the fastest axons to this muscle In a second series of 5 expts. as many motor axons as possible with conduction velocities above 60 m/s were stimulated Zones of glycogen depletion were found in 19 of the 47 examined spindles. They affected chain fibres in about half of the instances and bag fibres in the others. As this latter location is characteristic of slow dynamic β axons, it was concluded that both slow and fast β axons occur regularly in the motor supply to tenuissimus. β-innervation is present in at least 40 % of tenuissimus spindles with almost no convergence of fast and slow β axons onto the same spindle

Key words: skeleto-fusimotor innervation, β axons

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1. Conduction velocities of the motor axons [illegible] in the 5 expts. of the first series. [illegible] stimulated axons are represented by filled [illegible]angles.

examined spindles and the number of spindles in which glycogen depletion was found [illegible] it is [w]ell-known that the motor supply to tenuissimus does not comprise purely [illegible]motor axons [w]ith conduction velocities above 50 m/s (Kuffler, Hunt and Quilliam [19]51) and since there was no γ axon among the stimulated axons, intrafusal glycogen [de]pletion can in every case be considered evidence of fast β innervation.

Depleted intrafusal muscle fibres were observed in 16 spindles out of 46 examined (34 [illegible]). [M]ost of them were chain fibres as previously reported for the peroneus tertius muscle [(B]arker *et al.* 1977). A schematic reconstruction of one of the depleted spindles is [il]lustrated in Fig. 2. The longest chain fibre was depleted in both poles, mainly in its [ex]tracapsular portions. This chain fibre extends 1 mm beyond the end of the capsule at [on]e pole and even further at the other: it is therefore a "long chain" according to the criterion [o]f Barker *et al.* (1976 [illegible]).

TABLE 1. Number of tenuissimus spindles supplied by skeleto-fusimotor axons with conduction velocity higher than 85 m[/s]

Exp. No.	Number of stimulated motor axons (range of conduction velocities)	Number of examined spindles	Number of spindles [w]ith zones of glycogen depletion
1	16 (90–112 m/s)	13	4
2	18 (85–112 m[/s])	10	2
3	9 (85–107 m[/s])	7	2
4	16 (90–113 m/s)	6	3
5	13 (90–105 m/s)	10	5
Total		46	16

The essentials of the experimental methods were similar to those fully described by Barker et al (1977). All the hind limb muscles were denervated except the tenuissimus whose nerve was dissected for 10–15 mm and mounted on a recording electrode. The second electrode was placed in contact with the preparation at some distance from the tenuissimus. This made possible the recording through the same channel of the nerve and muscle action potentials elicited by the stimulation of motor axons in ventral roots.

The L7 and S1 ventral roots, which supply the tenuissimus, were split into small filaments and the conduction velocities of all motor axons to this muscle, γ as well as α, were measured. Only those filaments containing axons with conduction velocities above either 85 m/s (in the first series) or 60 m/s (in the second series) were kept for stimulation. All the filaments with slower axons were severed as close as possible to the dura. In order to facilitate the elimination of filaments containing γ axons, a reversible lignocaine block of nerve conduction as described by Barker et al. (1977) was used during the preparation of ventral root filaments in 3 expts. of the first series and 2 expts. of the second series.

The number of axons identified in each expt ranged from 9 to 21 (average 16) for the α axons and from 7 to 14 (average 9) for the γ axons.

After completion of ventral root exploration all the prepared filaments were mounted on a multi-wire stimulating electrode so as to be simultaneously excited. The nerve block was removed by washing the preparation with Ringer's solution and within an hour the conduction appeared fully restored in all the axons.

The regime of stimulation used to elicit glycogen depletion was similar to that used by Barker et al. (1977; see also Barker et al. 1977). Stimulation at 30/s was applied for one minute while the blood supply to the muscle was reduced by pulling on the external iliac artery with a rubber band. Stimulation was stopped for the following minute while a normal blood flow was allowed through the limb. This sequence was repeated 16 times without interruption and the muscle was excised during the final period of stimulation and blood occlusion.

The nerve action potentials were constantly monitored during stimulation in order to make certain that no stray γ axon was excited together with the selected axons, as may occur when γ axons recover from damage suffered during ventral root splitting. The absence of γ stimulation was finally checked by recording action potentials of the stimulated axons from the nerve stump after removal of the muscle.

A few seconds after excision, the muscle was quenched in isopentane cooled to −160 C. It was then stored at −30°C. The following day it was divided into portions that were cut frozen in serial transverse sections 25 μ thick. The sections were stained for glycogen using the PAS reaction and examined for glycogen depletion. The total length of muscle sectioned in each experiment (38 to 61 mm) represented from one quarter to one half of the whole muscle.

Serial reconstructions of spindles showing glycogen depletion were drawn in order to map the depleted zones in the intrafusal muscle fibres. The 3 types of fibre (bag_1, bag_2 and chain fibres) were identified on the basis of length, diameter, equatorial association and glycogen content, according to the criteria established by Banks, Harker and Stacey (1977). In a few instances, intrafusal muscle fibres which appeared paler than normal were observed suggesting that glycogen might have been uncompletely depleted in them. Such zones occurring in bag_1 fibres will be reported but in the final counts only those fibres showing zones of complete depletion were considered as being β-innervated.

Results

Spindles innervated by "fast" β axons in tenuissimus

The conduction velocities of the motor axons to tenuissimus identified in each experiment of the first series are shown in Fig. 1. All the stimulated axons in this series had conduction velocities above 85 m/s (expts. 2 and 3) or 90 m/s (expts. 1, 4 and 5). Their number ranged from 9 to 18 which represented, on average, 93 % of the axons with conduction velocities superior to 90 m/s present in the muscle nerve.

In each expt glycogen depletion was observed in several spindles after prolonged stimulation of a group of very fast conducting motor axons. For each of the 5 expts Table I gives the number of axons stimulated and their conduction [illegible]es, the number

Distal Pole — Proximal Pole

b1 b2 ch

1 2 3 4 5

3 Summary of the glycogen depletion elicited in 16 ... spindles by stimulating groups of motor ... with conduction velocities higher than 85 m/s. ... row represents ... spindle. The numbers on the left ... the different expts. Medium-diameter circles ... light shading represent non-depleted poles of bag, ..., large circles with coarse shading those of bag, ..., and small filled circles those of chain fibres. In ... spindle chain fibres are arranged in order of de... length. Fibres that contained ... zone of glycogen ... are represented by open circles. Open circles ... question mark represent bag, fibres that showed ... of presumed partial depletion (see text).

...cted two fibres which were the two longest chain fibres in two cases and the longest and ...th longest chain fibres in the other. It was also the longest chain fibre that was involved ... the 3 poles where bag fibres were depleted in addition to chain fibres. In summary ...henever a spindle pole showed chain fibre depletion, the longest chain fibre was affected ...e also Harker *et al.* 1977).

Of the 16 depleted spindles, only two were depleted in both poles and all the affected ...bres ...ere depleted in one zone only. I... total 9 spindles had a single ...one of depletion ...d 7 had two zones. The mean length of the depleted zones in the ...long chain... or in the ...ngest chain was 533 μm (for 16 zones ranging from 225 to 1 500 μm). The lengths of the depleted zones located on other chain fibres were 200, 250 and 300 μm respectively. For ...he 3 zones located on bag₁ fibres they were 325, 450 and 500 μm and for the 2 zones ...ocated on bag₂ fibres 125 and 1 500 μm respectively.

Assessment of the total number of β-innervated tenuissimus spindles

For the second series of experiments, cats could not be chosen randomly as was done for the first series, because axons in the 60–80 m/s conduction ...elocity range are often absent from the motor supply to cat tenuissimus. In such cases the lack of slow-conducting ...xons could be inferred first from the shape of the tenuissimus nerve ...ction potential recorded on stimulation of L7 and S1 ventral roots prior to any splitting. It was confirmed afterwards during ...entral root examination, as can be seen in Fig. 1 where histograms 1, 4 and 5 show gaps in this range. I... fact, 7 out of 14 examined cats showed similar gaps. Since the principle of the experiments implied the stimulation of as many as possible motor ...xons with conduction velocities covering the whole range of 60 to 110 m/s, it appeared

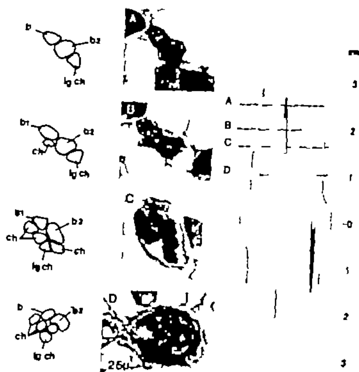


Fig. 2. Glycogen depletion elicited in a tenuissimus spindle by the simultaneous stimulation of 11 motor axons with conduction velocities higher than 85 m/s (expt. 2). *Left*. Microphotographs of spindle transverse sections stained to demonstrate glycogen (PAS method). In A the level of sectioning is near the tip of the spindle pole: the bag_1 (b1) and bag_2 (b2) fibres retain medium levels of glycogen while that of the long chain (lg ch) fibre is low. In B the level of sectioning is still extracapsular: the long chain is depleted, whereas the other fibres show normal levels of glycogen, i.e. medium for bag_1, medium/high for bag_2 and high for chain (ch) fibre. In C the level of sectioning is intracapsular: the long chain is depleted, whereas the bag fibres and the 3 other chain fibres show normal levels of glycogen. In D the level of sectioning is intracapsular and the long chain has recovered a normal content of glycogen. *Right*. Schematic reconstruction of the spindle showing the pattern of glycogen depletion. The light-shaded, coarse-shaded and filled intracapsular fibres are the bag_1, bag_2 and chain fibres, respectively. The blank stretches on the long chain indicate the zones of glycogen depletion. The dashed lines show the levels of transverse sections. The scale applies only to the length of the intrafusal muscle fibres.

The patterns of depletion observed in the 16 spindles are summarized in Fig. 3. These spindles contained a total of 32 bag fibres (one of each type per spindle) and 73 chain fibres (mean chain fibre complement 4.5 per spindle). In 11 spindles, only chain fibres were depleted. In 2 other spindles the bag_1 fibre was depleted in addition to chain fibres and in another spindle the bag_1 and a chain fibre were affected together. In the remaining 2 spindles (both from the same experiment) the bag_1 was the only fibre showing a zone of complete depletion but in the portion of the bag_2 fibre that lay side-by-side with the depleted zone, the glycogen content dropped to an extremely low level suggesting that some activation of the bag_2 fibre also might have occurred. In total, zones of complete depletion were found in 18 spindle poles, with chain fibres being involved in 16 poles, bag_1 fibres in 3 and bag_2 fibres in 2.

In the 16 poles with chain fibre depletion the distribution of depleted zones was as follows. In 13 poles a single chain was depleted which was invariably either a long chain (6 poles had such a fibre) or the longest chain fibre present. In 3 [illegible] oles, depletion

5. Summary of the glycogen depletion elicited in 19 tenuissimus spindles by stimulating groups of motor axons with conduction velocities higher than 60 m/s. Conventions as for Fig. 3. In expt. 6 the distal poles of two spindles were lost.

spindles. For the reason already mentioned—absence of purely fusimotor axons in the conduction velocity range—the depleted spindles may be considered as being β-innervated. In 17 of the 19 spindles both poles could be examined, but in 2 spindles, only one pole was available because of damage caused by division of the frozen muscle into small portions prior to sectioning. The patterns of depletion observed in these 17 whole spindles and 2 half spindles are summarized in Fig. 5. The 17 whole spindles contained a total of 34 bag fibres (one of each type per spindle) and 77 chain fibres (mean chain fibre complement 4.5 per spindle). The half spindles contained a total of 4 bag fibre poles and 7 chain fibre poles. In 9 whole spindles and in one half spindle, depletion occurred only in chain fibres. In 7 other whole spindles the bag₁ was the only depleted fibre. In each of the remaining whole spindle and half spindle the bag₁ and a chain fibre were affected together. It should also be mentioned that in 3 spindles the bag₂ fibre showed a zone of partial depletion, i.e. a zone of low glycogen content, which had the same length and the same location as the totally depleted zone of the neighbouring bag₁ fibre (in two spindles) or chain fibre (in the third spindle). In total, zones of complete depletion were found in 21 spindle poles with chain fibres being involved in 13 poles and bag₁ fibres in 10.

In keeping with previous observations (see the first series of expts. See also Harker *et al.* 1977) however, when a spindle pole showed chain fibre depletion it affected the longest of chain fibres, which actually was a long chain, in 5 cases. In 2 spindle poles, depletion affected the second longest chain fibre in addition to the longest one.

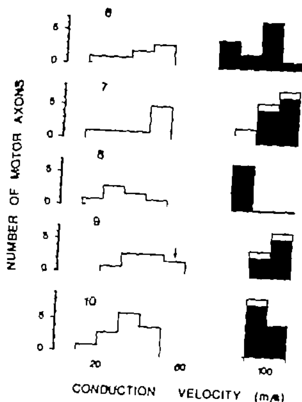


Fig. 4 Conduction velocities of the motor axons to tenuissimus in the 5 expts. of the second series. The stimulated axons are represented by filled rectangles. In histogram 9 the arrow points to 2 non-stimulated axons with conduction velocities between 50 and 60 m/s

necessary to select those cats whose tenuissimus was supplied also by some motor axons in the lower end of the range (Fig. 4). The selection probably explains the difference between the distributions of conduction velocities in the two series of experiments. The average proportion of axons having a velocity higher than 90 m/s was 85% and 65% in the first and second series respectively (compare Fig. 4 with Fig. 1). Whether this sampling has interfered with the results will be considered in the discussion.

Table II gives the number and conduction velocities of the stimulated axons, the number of examined spindles and the number of spindles in which glycogen depletion was found for each of the 5 expts. in this second series. The number of stimulated axons represented on average 92% of the axons with conduction velocities superior to 60 m/s present in the muscle nerve. Intrafusal depletion was observed in 19 (40%) of the 47 examined

TABLE II Number of tenuissimus spindles supplied by skeleto-fusimotor axons with conduction velocity higher than 60 m/s.

Exp. No.	Number of stimulated motor axons (range of conduction velocities)	Number of examined spindles	Number of spindles with zones of glycogen depletion
6	14 (70–109 m/s)	9	4
7	15 (60–109 m/s)	7	3
8	9 (60– 89 m/s)	8	2
9	11 (70–107 m/s)	11	4
10	17 (60–109 m/s)	12	6
Total		47	19

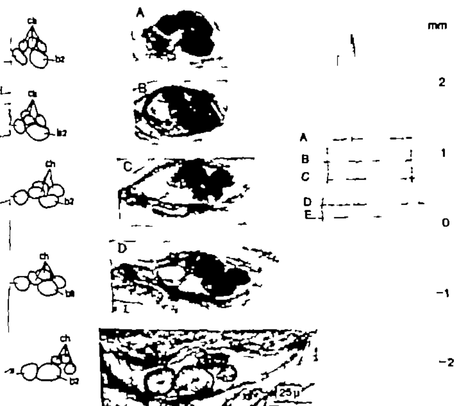


Fig. 6B

B Spindle with bag₁ fibre depleted in both poles. *Left* The microphotographs of spindle transverse sections show that the bag₁ fibre has normal glycogen level (medium) in A, which drops to low in B. The bag₁ fibre depleted in C and again low in D. The section in E is very near the equator so that all the intrafusal fibres appear paler than at polar levels. *Right* schematic reconstruction of the spindle showing the zones of glycogen depletion in both poles of bag₁ fibre. Dashed lines indicate the levels of transverse sections.

... in the longest chain fibre was 480 μm (for 12 zones ranging from 150 to 950 μm). The length of the two zones located on the second longest chain fibres were 200 and 375 μm respectively. The mean length of the depleted zones in the bag₁ fibre was 431 μm (for 8 zones ranging from 225 to 725 μm).

The main difference between the results of the two series of expts. lies in the intrafusal distribution of the zones of glycogen depletion. Table III shows that in all the expts. of the second series except one (n° 9), spindles with only chain depletion and spindles with only bag₁ depletion were found in the same muscle. Schematic reconstructions of two such spindles are shown in Fig. 6. In spindle A the pattern of depletion observed in the "long chain" is quite similar to the patterns observed in the first series of expts. (see also Harker et al. 1977), while the bag₁ depletion in spindle B compares with the patterns of depletion produced by stimulation of slow dynamic β axons (see Fig. 2 in Barker et al. 1977). It is

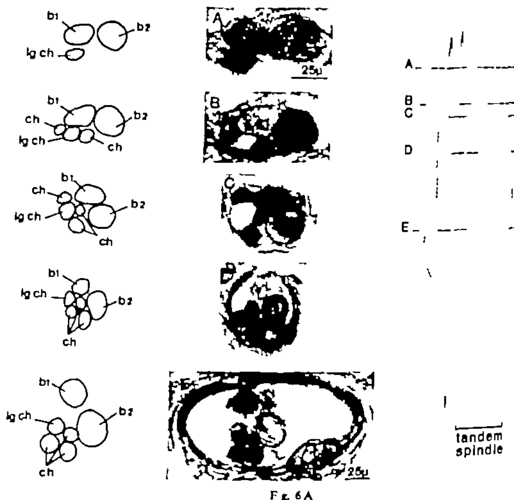
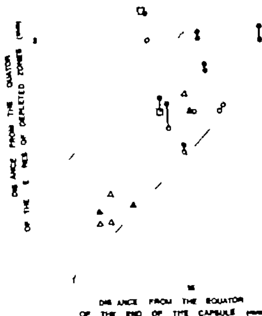


Fig. 6A

Fig. 6. Zones of glycogen depletion elicited in two different spindles from the same tenuissimus muscle by the simultaneous stimulation of 17 motor axons with conduction velocities higher than 60 m/s (expt 18). Same conventions as for Fig. 2.

A. Spindle with a long chain depleted in both poles. *Left*. Microphotographs of spindle transverse sections stained to demonstrate glycogen (PAS method). The scale in A is also valid for B, C and D. In A the level of sectioning is near the tip of the spindle pole; the levels of glycogen are medium for the bag fibres and high for the long chain fibre. In B the level of sectioning is intracapsular: the level of glycogen in the long chain fibre has dropped to medium/low; the bag fibre is medium; the bag fibre is medium high and the two other chain fibres are high. In C the level of sectioning is near the limit of the capsule; the long chain is depleted whereas the 3 other chain fibres are normally high. Note that the levels of glycogen are now medium/high and medium in bag and bag fibres respectively. In D the level of section is intracapsular: the long chain fibre is still depleted and the other chain fibres are normally high. The levels of glycogen in bag and bag fibres have reversed to medium/low and medium/high respectively. Note the general reduction of intrafusal muscle fibre diameters. In E the level of section is juxta-equatorial; the long chain has recovered a medium/high level of glycogen. Note the association of bag and chain fibres. *Right*. Schematic reconstruction of the spindle showing the zones of glycogen depletion in both poles of the long chain fibre. Dashed lines indicate the levels of transverse section. The bag and 2 chain fibres passed to another spindle linked in tandem to this spindle.

Of the 17 whole spindles only 2 were depleted in both poles, with the bag fibre being involved in one spindle and the "long chain" in the other. As in the first series, all the affected fibres were depleted in one zone only. 12 of the whole spindles had a single zone of depletion and 7 had two zones. The mean length of the depleted z[illegible] long chain



7. Distances from the spindle equator of centres of 26 zones of glycogen depletion (mm) plotted against the distance from spindle equator of the limit of the capsule (mm). Open circles represent zones of glycogen depletion located in chain fibres, triangles, zones located in bag$_1$ fibres and open squares, zones located in bag$_2$ fibres. Symbols linked by vertical line represent zones of glycogen depletion located in different fibres within the same spindle. The dashed lines indicate positions 0.5 mm from the limit of the capsule, either intracapsular (lower line) or extracapsular (upper line).

spindles in which chain fibres were depleted. Altogether the positions of the depleted spindles may be explained by assuming that in most instances there was either a single fast β axon or a single fast β axon plus a single slow β axon among the stimulated axons.

Discussion

The present experiments show that in tenuissimus muscle fast skeleto-fusimotor axons with conduction velocities above 85 m/s participate in the innervation of spindles and specifically of the longest intrafusal chain muscle fibres. The intrafusal patterns of glycogen depletion produced by fast β axons in tenuissimus were roughly similar to those found in peroneus tertius by Harker *et al.* (1977). Fast β axons occur regularly in the motor supply to tenuissimus since they were found in all expts. of both series. Since slow dynamic β axons have been previously demonstrated in tenuissimus (Barker *et al.* 1971; Emonet-Dénand *et al.* 1975; M. Williams 1975; Barker *et al.* 1977; Boyd *et al.* 1977) the first series of expts. suggests that both types of skeleto-fusimotor innervation may be present in this muscle. The actual coexistence of fast and slow β axons in the same muscle was demonstrated in the expts. of the second series.

Spindles showing the typical feature of slow β innervation, namely bag$_1$ fibre depletion (Barker *et al.* 1977), were found in all expts. of the second series except one (n. 9). However this exception does not prove that slow β axons to tenuissimus are inconstant, because histogram 9 in Fig. 4 shows that in this expt. 2 motor axons with conduction velocities

TABLE III. Intrafusal distribution of zones of glycogen depletion.

Exp No	Number of spindles in which depletion was located on			
	chain fibres only	chain fibres + bag$_2$ fibre	chain fibres + bag fibre	bag fibre only
1	2			2
2	2			
3	2			
4	2	1		
5	3	1	1	
Total	11	2	1	2
6	1		2	1
7	1			2
8	1			1
9	4			
10	3			3
Total	10		2	7

thus very likely that in the present sample the spindles showing bag$_1$ fibre depletion were innervated by slow β axons.

It is generally accepted—although not proven—that each zone of glycogen depletion found in intrafusal muscle fibre lies around the motor ending of the stimulated axon. The positions of the depleted zones might thus indicate the sites of termination of β axons. In Fig. 7 the distances from the spindle equator of the centres of the zones of glycogen depletion are plotted against the distance from the equator of the limit of the capsule in each spindle pole. Results from both series of experiments have been pooled showing that nearly all the centres of depleted zones were located within half millimeter from the end of the capsule either inside or outside the capsule sleeve. Similar locations of the zones of glycogen depletion elicited by stimulation of fast β axons in peroneus tertius spindles have been reported by Harker *et al.* (1977).

The number of β axons that were stimulated in either series of expts. is difficult to estimate for the following reasons. The only criterion for selecting the axons to be stimulated was their conduction velocity but it was impossible to ascertain that all the axons in the chosen range of conduction velocities were actually identified and prepared for stimulation. Table I and II compared to Fig. 1 and 4 show that there was no correlation between the proportion of stimulated axons and the proportion of depleted spindles. Moreover it is known that a single slow dynamic β axon can supply 4 spindles (Barker *et al.* 1977), but the average number of spindles innervated by a single fast β axon is unknown. However the number of β axons stimulated in the present expts. may be indirectly estimated from the position of depleted spindles in the examined portions of muscles. Fig. 8 shows that in experiment 5 for instance, the five depleted spindles were found within a portion of muscle inferior to 25 mm in length. Such close grouping of depleted spindles is rather suggestive of their being innervated by a single fast β axon. In expts. 7 and 10, spindles with bag fibre depletion also appear in close grouping and at some distance from the

s However since some spindles with bag₁ fibre depletion were found in expts. 1 and 5 Table III) the possibility should not be overlooked that some dynamic β axons may have been stimulated. Histograms 1 and 5 in Fig. 1 show wide gaps in motor axons [illegible] velocities between 50 and 80 m/s which makes not unlikely the presence of some β axons in the higher range of conduction velocity in these two experiments. [illegible] fast-conducting dynamic β axons to tenuissimus ha e already been observed since in [illegible] sample studied by Mc William (1975) the conduction velocities ranged from 63 to [illegible] m/s

The possibility of dynamic β axon activation in some experiments of the first series [illegible] also account for the fact that the intrafusal distribution of fast β axons appears less [illegible] in tenuissimus than in peroneus tertius. In the latter muscle glycogen depletion was [illegible] to chain fibres in 90 % of the depleted spindle poles (Harker *et al* 1977) as against [illegible] in the present sample. In any case, the specific innervation of the longest chain fibre [illegible] an intriguing feature of fast β axons. Their intrafusal distribution suggests that [illegible] γ exert static actions on spindle sensory endings but their selectivity for chain fibres [illegible] with the non-selective distribution of static γ axons (see the discussion in Harker *et al.* 1977)

A further question is whether the observed patterns of glycogen depletion fully account [for] the intrafusal distribution of the stimulated axons. In their γ-depletion study Barker *et al.* [19]76b) noted that complete depletion of chain fibres sometimes appeared difficult to [ach]ieve. This might also apply to bag₁ fibres which in the present sample showed more [in]stances of partial depletion (expts. 1 6, 9 and 10) than of complete depletion (expts. [3] and 5). Dynamic β axons only exceptionally depleted bag₂ fibres (Barker *et al* 1977) and [static] β axons to peroneus tertius were never observed to do so (Harker *et al* 1977). But [ev]idence has not been provided so far that bag₂ fibres might be deprived of β innervation. [On] the contrary the p₁ type of intrafusal motor end plates, which are considered as the [termi]nations of β axons (Barker Stacey and Adal 1970), have been found in almost equal [p]roportions on both types of intrafusal bag fibres in various hind-limb muscles of the [ra]bbit (Barker and Stacey 1970). Moreover the stimulation of static γ axons quite readily [pro]duced zones of glycogen depletion in bag₂ fibres (Barker *et al.* 1976). Since fast β [a]xons are probably static, it is surprising that when activated they should prove unable to deplete bag₂ fibres.

This investigation is supported by grants from the Institut National de la Santé et de la Recherche Médicale (AT 76-61) and the Fondation pour la Recherche Médicale Française.

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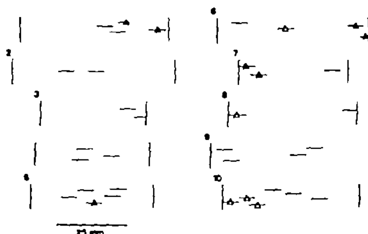


Fig. 8. Position of depleted spindles in the examined portion of tenuissimus muscles. The numbers indicate the different expts. Each block represents the length of the portion of muscle examined. Depleted spindles are represented by horizontal bars, those in which zones of glycogen depletion were located on the bag$_1$ fibre are marked by open triangles.

between 50 and 60 m/s were not stimulated (see arrow) and the possibility cannot be excluded that these axons were skeleto-fusimotor. It is therefore likely that slow β axons occur in tenuissimus as regularly as fast ones. In fact, if expt. 9 is removed from the results of the second series the total proportion of depleted spindles in the sample (15 out of 36 spindles, see Table III) comes out as 42% with approximately equal numbers of fast and slow β-innervated spindles (*i.e.* 6 spindles with only chain depletion and 7 with only bag depletion). The 2 spindles in which zones of glycogen depletion were located in both bag and chain fibres might be alloted to either type of β innervation since it is difficult to decide whether they represent occasional variations from regular patterns—such variations were observed in both of the glycogen-depletion studies of slow and fast β innervation (Barker *et al.* 1977; Harker *et al.* 1977)—or whether they are instances of slow and fast β axons converging onto the same spindle. The small number of spindles showing this pattern of depletion indicates that, for unknown reasons, such convergence is rare. Allowing for this uncertain convergence, the proportion of slow β-innervated spindles in the second series of expts. (n. 9 excluded) is at most 25% of the total sample and that of the fast β-innervated spindles is at most 22%.

However, this proportion of tenuissimus spindles supplied by slow β axons (25%) is probably still an underestimate due to the fact that the lower limit of conduction velocity for the stimulated axons was set at 60 m/s in these expts. It is known that the slowest dynamic β axons can be found in the γ range of conduction velocities (Bessou *et al.* 1965; Emonet-Dénand *et al.* 1975) but, if present in the studied muscles, such dynamic β axons would have been excluded from the group of stimulated axons. The probability of very slow β axons occurring in our sample is not negligible because of the sampling bias whose effect appeared to shift down by about 10 m/s the whole range of conduction velocities of motor axons to tenuissimus (compare Fig. 4 with Fig. 1). The assumption that the proportion of slow β-innervated tenuissimus spindles might be superior to 25% is further supported by the findings of Mc William (1975) who reported a proportion of 30% and considered it as a slight underestimate.

The assessed porportion of tenuissimus spindles innervated by fast β axons in the second series of expts. (22%) does not compare with the 34% depleted spindles found in the first

Acta physiol. scand. 1978. 103. 299–307

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Bronchial and cardiovascular actions of prostaglandin endoperoxides and an endoperoxide analogue

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Received 2 January 1978

Abstract

Hedqvist, P., K. Strandberg and M. Hamberg. *Bronchial and cardiovascular actions of prostaglandin endoperoxides and an endoperoxide analogue.* Acta physiol. scand. 1978. 103. 299–307.

Effects of PGG_2, PGH_2 and the endoperoxide analogue, EPA, on bronchial and vascular smooth muscle were studied in vivo and in vitro. In the cat PGG_2, PGH_2 and particularly EPA proved potent stimulators of airway resistance, and they were all significantly more active than $PGF_{2\alpha}$. They also increased pulmonary vascular resistance but EPA alone was more active than $PGF_{2\alpha}$. In guinea pigs EPA was 90–190 times more active than $PGF_{2\alpha}$ in increasing tracheal insufflation pressure. Human bronchi contracted in response to PGG_2, PGH_2 and EPA. PGG_2 and PGH_2 were equiactive with $PGF_{2\alpha}$ and EPA was more than 300 times as potent. PGG_2 and PGH_2 lowered systemic arterial blood pressure and increased heart rate in the cat. They were as active or more active than PGE_2. EPA, on the other hand, resembled $PGF_{2\alpha}$ in producing biphasic changes of blood pressure and heart rate or decrease of blood pressure and bradycardia in guinea pig and cat. The results obtained in this study indicate that PGG_2, PGH_2 and EPA are potent stimulators of bronchial and pulmonary vascular smooth muscle. The data are also consistent with the view that a significant proportion of effects resulting from prostaglandin generation in the lung is due to prostaglandin endoperoxides rather than primary prostaglandins.

The prostaglandin (PG) endoperoxides, PGG_2 and PGH_2, intermediates in the biosynthesis of PGE_2, $PGF_{2\alpha}$ and thromboxane A_2 (TxA_2) (Hamberg and Samuelsson 1973, Nugteren and Hazelhof 1973, Hamberg *et al.* 1975 b), have been shown to be potent constrictors of airway and vascular smooth muscles (Hamberg *et al.* 1975). However, due to the rapid conversion of the endoperoxides into their endproducts it was difficult to assess the quantitative or even qualitative actions of the endoperoxides *per se*. In this communication we report the actions of PGG_2, PGH_2, the synthetic stable endoperoxide analogue, 15(S)-hydroxy-9α,11α-(epoxymethano)prosta-5,13-dienoic acid (EPA), as well as some degradation products of the endoperoxides on the respiratory and cardiovascular systems in cats and guinea pigs, and on isolated human bronchi. A preliminary account of part of the results has been presented elsewhere (Hedqvist *et al.* 1974).

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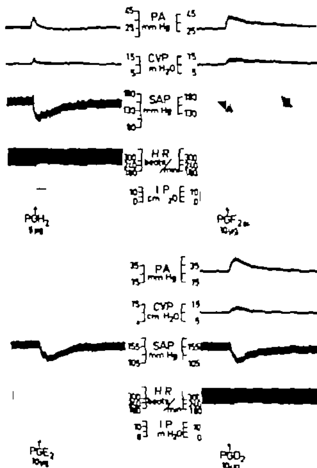


Fig. 1 Effects of intravenously administered PGD2, PGE2, PGF2α and PGH2 on pulmonary arterial pressure (PA), central venous pressure (CVP), systemic arterial pressure (SAP), heart rate (HR), and tracheal insufflation pressure (IP) cat.

when given i.a. and PGG and PGH equally active on i. and i.a. administration (Table III). $PGF_{2\alpha}$ affected the systemic arterial blood pressure in a different manner. In most cases it caused a biphasic response, either an initial drop followed by a moderate and more longlasting increase (Fig. 1), or the reverse pattern. However in some expts. the effect of

Table 1 Relative activity of PGD_2, PGG_2, PGH_2, EPA and $PGF_{2\alpha}$ on pulmonary arterial pressure (P_{PA}) and tracheal insufflation pressure (P_{TR}) in cats. Mean values ± S.E.

	PGD_2	PGG_2	PGH_2	EPA	$PGF_{2\alpha}$
P_{PA}	0.54 ± 0.16 6 P 0.05	1.21 ± 0.40 5	1.08 ± 0.27 9	33.9 ± 8.9 15 P 0.01	1.0
P_{TR}	1.33 ± 0.36 12	2.33 ± 0.59 12 P 0.05	4.43 + 0.54 17 $P < 0.02$	44.9 + 10.9 15 $P < 0.01$	1.0

Methods

Cats of either sex (2.5–4.0 kg) were anesthetized with sodium pentobarbitone (30 mg/kg) i.p. and supplementary doses were administered i.v. as required to maintain anesthesia. The trachea was cannulated and artificial respiration was administered with a Harvard small animal respirator. Spontaneous breathing was precluded with gallamine triethiodide (Flaxedil®) (1 mg/kg every 30 min). Changes in tracheal insufflation pressure were recorded by means of a Statham pressure transducer (P23 BC) connected to the side arm of the airway circuit (inlet), and a Grass Model 5D polygraph. The main pulmonary artery was approached through a leftward thoracotomy and a thin indwelling polyethylene catheter was placed in the artery. Polyethylene catheters were also inserted in the right femoral vein and artery and advanced to the abdominal part of v. cava and the aorta, respectively. Blood pressures were recorded with Statham pressure transducers (P23 BC for central venous pressure and P23 AC for pulmonary artery and systemic pressure) and the Grass polygraph. The heart rate was measured by the use of an ordinate meter triggered by the arterial pulse wave. Test agents were injected into the right jugular vein or the aortic arch.

Male albino guinea pigs (500–700 g) were anesthetized with sodium pentobarbitone (60 mg/kg i.p.) and were prepared for registration of tracheal insufflation pressure as previously described in detail (Strandberg and Hedqvist 1975). Briefly the animals were ventilated with a Palmer constant volume respirator (8 strokes/min, 4–6 ml/kg b.w.) and changes in insufflation pressure were measured with a Statham pressure transducer (P23 BC) connected to the tracheal cannula. Systemic arterial blood pressure and heart rate were monitored from the right carotid artery using a Statham pressure transducer (P23 AC). All parameters were recorded on a Grass 5D polygraph. At the beginning of the experiment heparin (300 IU/kg) was given into the right jugular vein. This route was also used for administration of test agents and anesthetic supplements.

Human bronchi were free-dissected from macroscopically healthy pulmonary tissue obtained in connection with surgery for pulmonary tumours. Helical strips of bronchi were either kept in 5 ml organ baths containing Tyrode's solution or superfused at a rate of 1.5 ml/min with Tyrode (conc. in mM: NaCl 136.7, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.5), maintained at 37°C and gassed with 5% CO_2 in O_2. Isometric recordings of the smooth muscle activity were obtained using a force displacement transducer (Grass FT 03) with a load of 0.5 g and a Grass 5D polygraph.

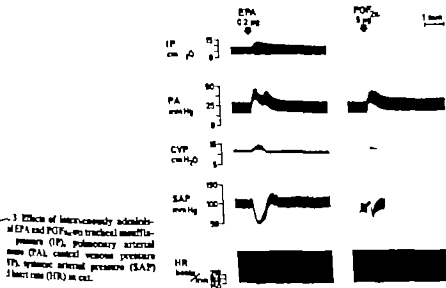
PGG_2 and PGH_2, prepared according to Hamberg *et al.* (1974), were kept in acetone (1 µg/µl) at −20°C and diluted with 0.9% NaCl immediately before use. The final concentration of acetone did not influence any of the studied parameters. PGD_2, PGE_2, $PGF_{2\alpha}$ as well as EPA (Bundy 1975) were generously supplied by Dr. J. Pike, Upjohn Co., Kalamazoo, Michigan.

Statistical analysis was performed according to Student's t-test for paired and unpaired data.

Results

Cat experiments

Fig. 1 illustrates some cardiovascular and bronchial effects of the endoperoxide PGH_2 and some of its possible degradation products. Upon injection into the right jugular vein PGH_2, PGD_2 and $PGF_{2\alpha}$ (5–10 µg) regularly increased pulmonary arterial pressure, central venous pressure and tracheal insufflation pressure, whereas PGE_2 in the same concentrations only slightly and inconsistently affected these parameters. Comparison of relative activities of endoperoxides and degradation products on pulmonary arterial pressure revealed that PGG_2, PGH_2 and $PGF_{2\alpha}$ were of the same potency and PGD_2 less active (Table I). On the other hand the rank order of potency on tracheal insufflation pressure was $PGG_2 = PGH_2 > PGF_{2\alpha} = PGD_2$ (Table I). Upon i.v. administration PGD_2, PGE_2, PGG_2 and PGH_2 all reduced systemic arterial blood pressure and increased the heart rate (Fig. 1, Table II). PGD_2 was less and PGH_2 significantly more effective than PGE_2. On the other hand PGE_2 was as active a vasodepressor as PGH_2 when the two compounds were given i.a. Comparison of i.v. and i.a. responses revealed that P[illegible] was more active



3 Effects of intravenously administ-
ed EPA and $PGF_{2\alpha}$ on tracheal insuffla-
pressure (IP), pulmonary arterial
(PA), central venous pressure
(), systemic arterial pressure (SAP)
heart rate (HR) in cat.

logue EPA was used. EPA doses of the order of 0.1–0.5 μg sufficed to markedly increase tracheal insufflation pressure and pulmonary arterial pressure. In the latter case a two peak response was often observed, suggesting recirculation of intact EPA (Fig. 3). Relative to GF EPA was 30–40 times more potent on tracheal insufflation pressure and pulmonary arterial pressure (Table I). As with $PGF_{2\alpha}$ EPA produced mixed effects on systemic arterial pressure. The most prevalent response was an initial drop followed by a moderate increase, although pure depressor or pressor responses were also seen. The effect of EPA on the heart rate was consistently that of a slight decrease.

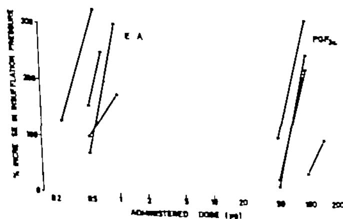


Fig. 4. Dose-response relations for intravenously administered $PGF_{2\alpha}$ and EPA on tracheal insufflation pressure in 4 guinea pigs.

TABLE II. Relative depressor activity of intravenously administered PGD_2, PGG_2, PGH_2 and PGE_2 on systemic arterial pressure in cats. Mean values ± S.E.

PGD_2	PGG_2	PGH_2	PGE_2
0.61 ± 0.12	1.71 ± 0.37	1.91 ± 0.38	1.0
n = 10	n = 7	n = 12	
$P < 0.05$	n.s.	$P < 0.05$	

TABLE III. Vasodepressor effect of a standard dose (10 µg) of PGE_2, PGG_2 and PGH_2 given either in the jugular vein (i.v.) or in the aortic arch (i.a.) in cats. D is presented as percent fall in blood pressure and given as means ± S.E. (N) where N is number of expts.

	PGE_2	PGG	PGH_2
i.v.	23.2 ± [illegible].0	27.6 ± 4.4	34.3 ± 3.[illegible]
	(8)	(7)	(10)
i.a.	31.0 ± 2.4	27.6 ± 4.1	31.6 ± 2.0
	(3)	(3)	(3)
	$P < 0.05$	n.s.	n.s.

$PGF_{2\alpha}$ was purely depressor. Independently of whether $PGF_{2\alpha}$ was pressor or depressor it consistently caused a slight and shortlasting decrease in the heart rate.

In some expts. administration of PGG or PGH was repeated after incubation of the compound in 0.9% NaCl or blood for 20 min at 37°C. This treatment almost abolished the effect of the compound on pulmonary arterial pressure and tracheal insufflation pressure and it reduced the effect on systemic arterial pressure, *i.e.* resultant effects closely resembling those of PGE_2 (Fig. 2).

In order to get more insight in effects of the endoperoxides *per se* the stable endoperoxide

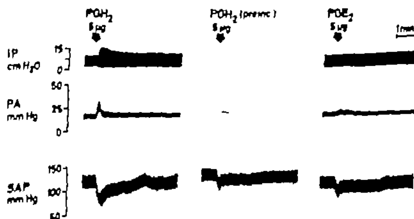
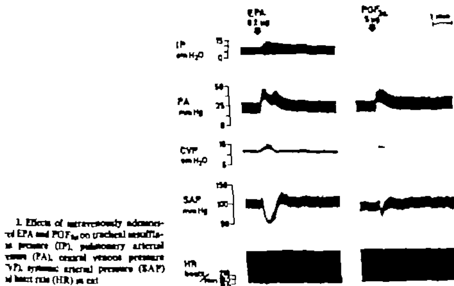


Fig. 2. Effects of intravenously administered PGH_2, PGH preincubated in 0.9% NaCl for 20 min at 37°C, and PGE_2 on tracheal insufflation pressure (IP), pulmonary arterial pressure (PA) and systemic arterial pressure (SAP) in cat.



1. Effects of intravenously administered EPA and $PGF_{2\alpha}$ on tracheal insufflation pressure (IP), pulmonary arterial pressure (PA), central venous pressure (CVP), systemic arterial pressure (SAP) and heart rate (HR) in cat

analogue EPA was used. EPA doses of the order of 0.1–0.5 μg sufficed to markedly increase tracheal insufflation pressure and pulmonary arterial pressure. In the latter case a two peak response was often observed, suggesting recirculation of intact EPA (Fig. 3). Relative to $PGF_{2\alpha}$ EPA was 30–40 times more potent on tracheal insufflation pressure and pulmonary arterial pressure (Table I). As with $PGF_{2\alpha}$ EPA produced mixed effects on systemic arterial pressure. The most prevalent response was an initial drop followed by a moderate increase, although pure depressor or pressor responses were also seen. The effect of EPA on the heart rate was consistently that of a slight decrease.

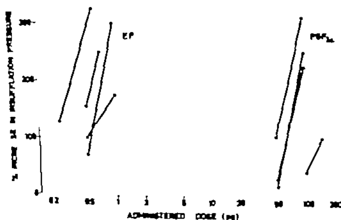


Fig. 4. Dose-response relations for intravenously administered $PGF_{2\alpha}$ and EPA on tracheal insufflation pressure in 4 guinea pigs

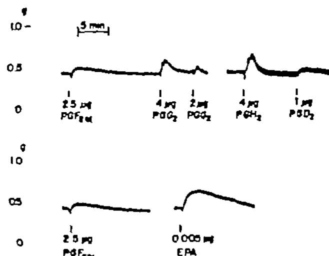


Fig. 5. Contractile effects of PGF, PGG_2, PGH, PGD_2 and EPA on a superfused human bronchial strip.

Guinea pig experiments

Previously it has been shown that the endoperoxides are 8 11 times more potent than $PGF_{2\alpha}$ in eliciting an increase in tracheal insufflation pressure in guinea pigs (Hamberg *et al* 1975 a) In the present expts. the endoperoxides were replaced by EPA and its effect on tracheal insufflation pressure was compared with that of $PGF_{2\alpha}$. Both compounds increased the tracheal insufflation pressure, but EPA was considerably more potent than $PGF_{2\alpha}$ (Fig. 4) Calculating the relative activity revealed that EPA was 187±47 (mean ±S.E., n=4) times more potent than $PGF_{2\alpha}$ in this respect. The effect of EPA on the cardiovascular system was similar to that of $PGF_{2\alpha}$, *i.e* a transient fall in systemic arterial blood pressure and bradycardia, followed by a more longlasting rise in pressure and tachycardia

Human bronchi in vitro

In Fig. 5 is illustrated the contractile effect of PGD_2, $PGF_{2\alpha}$, PGG_2, PGH and EPA on isolated human bronchi, superfused with Tyrode at a rate of 1.5 ml/min. PGD_2, PGG and PGH were approximately as potent bronchoconstrictors as $PGF_{2\alpha}$, whereas EPA was considerably more active in this respect. The relative contractile effects of the different compounds are shown in Table IV

In other expts. human bronchi were incubated in 5 ml Tyrode, and dose response curves for $PGF_{2\alpha}$ and EPA were determined by stepwise raising the concentrations of the compounds in the bath. Approximately parallel dose–response curves were obtained, but EPA was 92±45 (mean±S.E., n=7) times more active than $PGF_{2\alpha}$.

TABLE IV Relative contractile effects of PGD_2, PGG_2, PGH_2, EPA and $PGF_{2\alpha}$ on isolated human bronchi. Mean values ± S.E. Figures within brackets = number of expts.

PGD_2	PGG_2	PGH	EPA	$PGF_{2\alpha}$
1.2±0.2 (n=3) n.s.	1.2±0.4 (=4) n.s.	[illegible]±0.8 (=4) n.s.	569±146 (=5) P<0.01	1.0

Discussion

...e present study PGG and PGH were found to increase tracheal insufflation pressure ...n significantly more than did $PGF_{2\alpha}$, which is in harmony with previous observations ...e guinea pig (Hamberg *et al.* 1975 a). The endoperoxides also increased pulmonary ...ial pressure and they were of the same potency as $PGF_{2\alpha}$ in this respect. Änggård and ...ström (1963) reported that $PGF_{2\alpha}$ increased both tracheal insufflation and right ...ricular pressures in cats and attributed the bronchial effect of the compound to ...traction of bronchial smooth muscle or pulmonary congestion or a combination of ...factors. However because the effect of $PGF_{2\alpha}$ and endoperoxides on pulmonary ...rial pressure is similar the relatively higher potency of the endoperoxides on tracheal ...fflation pressure found in the present study is hard to explain in terms of pulmonary ...gestion. Moreover while $PGF_{2\alpha}$ caused bradycardia the endoperoxides consistently ...duced an increase in heart rate. Therefore, the endoperoxides should be considered ...at bronchoconstrictors *in vivo* just as they have been shown to be in different *in vitro* ...tems (cf. Hamberg *et al.* 1975 a).

PGD_2, PGE_2, PGG and PGH depressed systemic arterial blood pressure and meanwhile ...sed a moderate rise in heart rate, presumably secondary to the fall in blood pressure. ...ese compounds are depressors also in the rat (Armstrong *et al.* 1976), whereas in the guinea ...g PGD, and PGE_2 are depressors and the endoperoxides cause a triphasic change in blood ...ssure (Hedqvist *et al.* 1974 Hamberg *et al.* 1975 a). PGH was significantly more ...tent than PGE_2 when given by the route. This difference in activity was annulled ...en PGH_2 was preincubated in saline or when the two compounds were given i.a. ...oreover PGH was equally active on i. and i.a. administration, and PGE_2 more active ...en given i.a. than The difference in activity between the two compounds upon i. ...ministration could be explained by PGE_2 being partly metabolized during the passage ...rough the lung circulation (Ferreira and Vane 1967), and PGH leaving the lung circula... ...ion either intact or as an active metabolite, which could be PGE_2 but not PGD or ...$PGF_{2\alpha}$. Admittedly PGH caused a rise in pulmonary arterial and central venous pressures ...but the short duration of these effects makes it unlikely that pulmonary congestion should ...have affected significantly the vasodepressor activity of the compound.

The relative potency of PGE_2 and PGH as vasodepressor agents has been studied also in the rat (Armstrong *et al.* 1976). In this animal species PGE_2 was found to be 6 times more effective than PGH_2 in reducing systemic arterial blood pressure upon i.a. administration, whereas the two compounds were approximately equieffective when given i. Species differences may account for the dissimilar results in rat and cat.

Because the endoperoxides may be rapidly transformed into other compounds, chiefly PGE_2, $PGF_{2\alpha}$ and PGD (Hamberg and Samuelsson 1973 Nugteren and Hazelhof 1973) but also thromboxanes (Hamberg *et al.* 1975 b) their bronchial and cardiovascular effects were compared with those of the stable endoperoxide analogue EPA. EPA was found to be 30–40 times more potent than PGG or PGH in increasing bronchial and pulmonary vascular resistances in the cat. In the guinea pig EPA was near 200 times more potent than $PGF_{2\alpha}$ in causing an increase in tracheal insufflation pressure. The difference in

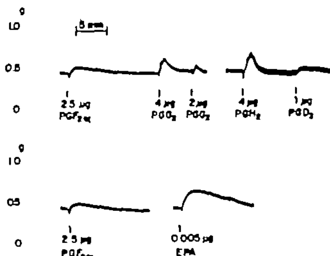


Fig. 5 Contractile effects of PGF2α, PGG2, PGH2, PGD2 and EPA on superfused human bronchial strip

Guinea pig experiments

Previously it has been shown that the endoperoxides are 8–11 times more potent than $PGF_{2\alpha}$ in eliciting an increase in tracheal insufflation pressure in guinea pigs (Hamberg *et al* 1975 a). In the present expts. the endoperoxides were replaced by EPA and its effect on tracheal insufflation pressure was compared with that of $PGF_{2\alpha}$. Both compounds increased the tracheal insufflation pressure, but EPA was considerably more potent than $PGF_{2\alpha}$ (Fig. 4). Calculating the relative activity revealed that EPA was 187±42 (mean ±S.E., n = 4) times more potent than $PGF_{2\alpha}$ in this respect. The effect of EPA on the cardiovascular system was similar to that of $PGF_{2\alpha}$, *i.e.* a transient fall in systemic arterial blood pressure and bradycardia, followed by a more longlasting rise in pressure and tachycardia.

Human bronchi in vitro

In Fig. 5 is illustrated the contractile effect of PGD_2, $PGF_{2\alpha}$, PGG_2, PGH and EPA on isolated human bronchi, superfused with Tyrode at a rate of 1.5 ml/min. PGD_2, PGG and PGH were approximately as potent bronchoconstrictors as $PGF_{2\alpha}$, whereas EPA was considerably more active in this respect. The relative contractile effects of the different compounds are shown in Table IV.

In other expts. human bronchi were incubated in 5 ml Tyrode, and dose-response curves for $PGF_{2\alpha}$ and EPA were determined by stepwise raising the concentrations of the compounds in the bath. Approximately parallel dose-response curves were obtained, but EPA was 92±45 (mean ±S.E., n = 7) times more active than $PGF_{2\alpha}$.

TABLE IV. Relative contractile effects of PGD_2, PGG_2, PGH_2, EPA and $PGF_{2\alpha}$ on isolated human bronchi. Mean values ± S.E. Figures within brackets number of expts.

PGD	PGG	PGH	EPA	$PGF_{2\alpha}$
1.2±0.2 (n = 3) n.s.	1.2±0.4 (n = 4) n.s.	2.2±0.8 (n = 4) n.s.	569±146 (n = 5) $P < 0.01$	1.0

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activity should be compared with our previous report that the endoperoxides are 1- times more potent than $PGF_{2\alpha}$ in this respect (Hamberg *et al* 1975a). Recently similar although less dramatic effects of EPA have been shown in the dog, inasmuch EPA was 4–8 times more potent than $PGF_{2\alpha}$ in causing a decrease in lung compliance and increase in lung resistance (Wasserman 1976). Furthermore another endoperoxide analog resembling EPA was approximately 10 times more active than $PGF_{2\alpha}$ in increasing arterial pressure in canine lung lobes perfused with blood or dextran (Kadowitz and Hyman 1977) The effects obtained with EPA therefore seem to indicate that endogenously formed endoperoxides are more potent on pulmonary vascular and bronchial smooth muscle than is obvious from their exogenous application. However in the cat EPA produced some cardiovascular effects, which differed from those obtained with endoperoxides biphasic changes in blood pressure and bradycardia rather than fall in pressure and tachycardia. Moreover in the dog EPA decreases and PGH increases blood flow in the mesenteric artery (Chapnick *et al* 1977). Therefore the possibility should not be overlooked that also in the pulmonary circulation EPA produces effects that cannot be accounted for by endoperoxides.

PGD_2, $PGF_{2\alpha}$ PGG PGH and EPA were all found to contract isolated human bronchi PGD_2, PGF PGG and PGH were approximately equiactive, whereas EPA demonstrated a 90–190 times higher potency Previously it has been shown that PGD_2 5 times and the endoperoxides 8–9 times more potent than $PGF_{2\alpha}$ in eliciting contraction of isolated guinea pig trachea (Hamberg *et al* 1975 a). The relatively lower activity of endoperoxides and PGD on human bronchi might reflect different patterns of reaction However the difference could also be due to a more rapid conversion of endoperoxides into their end products in human bronchi than in guinea pig trachea. Consistent with the latter view is the remarkable bronchoconstrictive effect of EPA compared with the endoperoxides.

Primary prostaglandins have been implicated in normal and disordered lung function notably bronchial asthma (*cf* Mathé *et al* 1977) Recent studies have shown, however, that PGG and PGH are 8–11 times and thromboxane A 500 times more potent than $PGF_{2\alpha}$ in increasing tracheobronchial insufflation presure in guinea pig (Hamberg *et al* 197 Svensson *et al* 1977) The present data demonstrating marked pulmonary actions of endoperoxides and an endoperoxide analogue in cats and guinea pigs *in vivo* and human bronchi *in vitro* add further weight to the concept that a significant proportion of biological effects occurring on activation of prostaglandin synthetase can be accounted for by endoperoxides and/or thromboxane A_2. Since these compounds are all potent bronchoconstrictors a role for the E prostaglandins with regard to airway patency might seem difficult to recognize. It is conceivable, however that conversion of endoperoxides into thromboxane A occurs only under certain conditions, and that the biological action of endoperoxides *per se* is balanced by their half life normally being short in comparison with that of PGE_2.

This study was supported by grants from the Swedish Medical Research Council (04X-434), from Swedish National Association against Heart and Chest Diseases and from Karolinska Institutet.

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Acta physiol. scand. 1978. 103 308–319

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The influence of supraspinal impulse activity on the intra-axonal transport of acetylcholine, choline acetyltransferase and acetylcholinesterase in rat motor neurons

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Received 2 January 1978

Abstract

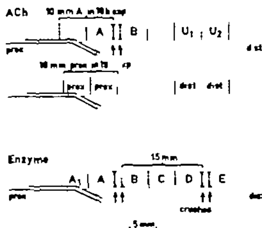
DAHLSTRÖM A., P.-O. HEIWALL, S. BÖÖJ and A.-G. DAHLLÖF *The influence of supraspinal impulse activity on the intra-axonal transport of acetylcholine, choline acetyltransferase and acetylcholinesterase in rat motor neurons* Acta physiol. scand. 1978 *103* 308–319

The effect of supraspinal impulse activity upon the intra-axonal transport of acetylcholine (ACh), AC[illegible]esterase (AChE) and [illegible]holineacetyltransferase (CAT) in rat sciatic nerve has been studied. A decrease[illegible] impulse activity was obtained by spinal cord transection (SCT) in the thoracic region 18 h, 6 days or [illegible] days before killing the rats. A[illegible] increased neuronal activity was obtained by exercising the rats [illegible] commercial rodent treadmill a couple of hours per day for 14 days. The amounts of substances [illegible] had accumulated in the sciatic nerve segments relative to [illegible] nerve crush performed 12 or 18 h. earlier we[illegible] used to calculate the intra-a[illegible]onal transport. The amounts of pro[illegible]imo-distally transported ACh decrease[illegible] markedly with time after the SCT[illegible] while th[illegible] proximo-distal transport of AChE-activity increase[illegible] Physical exercise appeared t[illegible] increase ACh-transport. Thus, i[illegible] put t[illegible] motor pericary[illegible] from supraspinal centres may regulate [illegible]tra-axonal transport from th[illegible] cell body of motor neurons into their axons.

Key words. Intra-axonal transport, rat motor neurons, acetylcholine, chol[illegible]nergic enzymes, spinal cord transection

In motor nerves of mammals a proximo-distal, intra-axonal transport of various substances takes place. Thus, labelled proteins have been found to be transported at rates varying between 2–3 mm/day and 400 mm/day (*cf* Ochs and Johnson 1969). The slow phase of transport mainly carries soluble proteins and microtubular and filamentous proteins while preferentially membrane-bound material is moving with the more rapid rates of transport (*e.g* Sabri and Ochs 1973) In agreement with this the soluble enzyme choline acetyltransferase (CAT) in rat motor nerves appears to be transported with the slow phase (Saunders *et al* 1973) while the acetylcholinesterase (AChE) which is membrane-bound (Kása *et al* 1973), is transported at rapid rates (*e.g* Lubińska and Niemierko 1971 Ranish and Ochs 1972)

Also the transmitter in motor nerves, acetylcholine (ACh), appears to be transported intra-axonally at rates of about 120 mm/day in the rat sciatic nerve (*e.g* Dahlström *et al.*



1 Schematic illustration of the mode of ... of the rat sciatic nerve, relative ... mm. Each crush was ... separated by 1 mm nerve.

... It is not known in what compartment this transportable ACh is located, but ... of the rather rapid rate of transport it is likely that this ACh (bout 15–20*' of ... total axonal ACh) may be bound within a membrane-limited structure (see discussion Dahlström *et al* 1974 b).

Changes in impulse activity of a neuron can influence the amounts of transported ..., whilst the rate of transport seems to be unaltered (*cf* reviews by Dahlström ..., Heslop 1975). Thus, increased impulse activity in adrenergic nerves causes an increase ... the amounts of transported noradrenaline and dopamine β-hydroxylase in the axons ... Kuo and McLean 1975) In the present study we have therefore investigated the effect ... both decreased and increased impulse activity on the intra-axonal transport of ACh, CAT ... AchE in rat sciatic nerve.

Materials and methods

... rats (200–250 g) of both sexes were used in all expts. *Decreased impulse activity* in the sciatic nerve ... was obtained by sectioning the spinal cord at the lower thoracic level 18 h, 6 days or 20 days prior ... death. All operative procedures were performed under ether anaesthesia. The bladder of the spinal cord ... (SCT) animals was emptied by exterior manipulations twice daily. This was found to be ... since all SCT animals showed marked initial hematuria, tending to plug the urethra if the bladder was not emptied regularly. This hematuria disappeared after couple of days. It as easier to keep ... spinalized rats healthy during longer postoperative periods, hich was the reason for using also ... rats, especially for the 20 days SCT study. Control animals were handled twice daily and housed ... the same room as the SCT animals.

An increased impulse activity as obtained by exercising the rats 2 3 h daily for 14 days in ... rodent treadmill (Quinton Instruments, Seattle Wash.). Controls were allowed to sta in the treadmill for an equivalent period of time but ithout moving the track. The exercise as mild at the beginning of the period and gradually increased in speed and duration (g Barnard and Peter 1971). ... rats showed large variations in exercise tolerance some had to be excluded from the experiment at an early stage due to poor capacity.

12 h or 18 h (for exercise expt) prior to sacrifice about half of the animals in each group ere reoperated under ether anaesthesia. The sciatic nerves bilaterally were exposed and nerve crushes (a.m. Lubinska ...) were made between a glass rod (0 5 mm) and silk suture (3 0).

For ACh assays the nerves were crushed at high level only The crush as included 2 crushes separated by 1–2 mm. 12 h after crushing 5 mm segments just proximal (A) and distal (B) to the site of

Acta physiol. scand. 1978. 103. 308–319

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The influence of supraspinal impulse activity on the intra-axonal transport of acetylcholine, choline acetyltransferase and acetylcholinesterase in rat motor neurons

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Received 2 January 1978

Abstract

DAHLSTRÖM, A., P.-O. HEIWALL, S. BÖÖJ and A.-G. DAHLLÖF. *The influence of supraspinal impulse activity on the intra-axonal transport of acetylcholine, choline acetyltransferase and acetylcholinesterase in rat motor neurons.* Acta physiol. scand. 1978. 103. 308–3[illegible]

The effect of supraspinal impulse activity upon the intra-axonal transport of acetylcholine (ACh), acetylcholinesterase (AChE) and choline acetyltransferase (CAT) in rat sciatic nerve has been studied. A decreased impulse activity was obtained by spinal cord transection (SCT) in the thoracic region 18 h, 6 days or [illegible] days before killing the rats. An increased neuronal activity was obtained by exercising the rats in a commercial rodent treadmill a couple of hours per day for 14 days. The amounts of substances which had accumulated in the sciatic nerve segments relative to a nerve crush performed 12 or 18 h earlier were used to calculate the intra-axonal transport. The amounts of proximo-distally transported ACh decreased markedly with time after the SCT, while the proximo-distal transport of AChE-activity [illegible]. Physical exercise appeared to increase ACh-transport. Thus, input to motor pericarya from supraspinal centres may regulate intra-axonal transport from the cell body of motor neurons into their axons.

Key words: Intra-axonal transport, rat motor neurons, acetylcholine, cholinergic enzymes, spinal cord transection

In motor nerves of mammals a proximo-distal, intra-axonal transport of various substances takes place. Thus, labelled proteins have been found to be transported at rates varying between 2–3 mm/day and 400 mm/day (*cf.* Ochs and Johnson 1969). The slow phase of transport mainly carries soluble proteins and microtubular and filamentous proteins while preferentially membrane bound material is moving with the more rapid rates of transport (*e.g.* Sabri and Ochs 1973). In agreement with this the soluble enzyme choline acetyltransferase (CAT) in rat motor nerves appears to be transported with the slow phase (Saunders *et al.* 1973) while the acetylcholinesterase (AChE), which is membrane-bound (Kása *et al.* 1973), is transported at rapid rates (*e.g.* Lubińska and Niemierko 1971, Ranish and Ochs 1972).

Also the transmitter in motor nerves, acetylcholine (ACh), appears to be transported intra-axonally at rates of about 120 mm/day in the rat sciatic nerve (*e.g.* Dahlström *et al.*

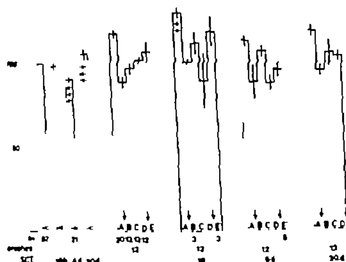


Fig. 3. CAT-activity expressed as percent of control uncrushed nerve in rat sciatic nerves at various times following spinal cord transection (SCT). Arrows indicate sites of crushes 12 h before dissection (see Fig. 1). Mean ± S.E. are given. 100% = 996 ± 43 pmol of ACh formed/hour/5 mm nerve. * indicates different from control A-segment, p < 0.01. + indicates different from control uncrushed nerve, p < 0.005.

...served after SCT although a tendency towards lower values were seen in 18 h and 4 days SCT-operated animals. In all groups, the distal 5 mm segment appeared to contain more ACh than the proximal segment.

In the 12 h crushed nerves of the control group the segment A contained 139 ± 4.4 pmol of ACh. After SCT the ACh in the A-segment was only 107 ± 6.1 pmol in 18 h operated animals and 90 ± 9.9 pmol 20 days after the spinal cord operation (Fig. 2). In the B-segment, just distal to the crushes, the ACh-content was somewhat lower in 18 h and 4 days SCT-rats, but normal or possibly supranormal in 20 days operated rats. In the C-segments, which were assayed for ACh to give some information on the "transportable" fraction of ACh, the ACh content was always somewhat lower (7%, 9%, 16% and 25% in 0 to 20 d SCT animals, respectively Fig. 2) than the corresponding control piece in uncrushed nerves (distal), but a significant decrease was obtained only in the 20 d SCT operated animals.

The amount of ACh transported to the A-segment from more proximal sites in the neuron by axonal transport can be obtained by the following calculation. $ACh_A = ACh_{transported} + ACh_{stationary}$. Since the B-segment contains $ACh_{post-transected} + ACh_{stationary}$ (Feldberg 1943), the calculation is reduced to $ACh = ACh_A - ACh_{B}$. This is indicated in Fig. 6. It can be seen that the rapid initial decrease in transported ACh is about 17% (18 h) and by 20 days the decrease was about 50% (Fig. 6).

CAT. In uncrushed nerves of control animals no difference in CAT-activity was observed along the nerve in the various segments A–E. The CAT-activity in the uncrushed nerves

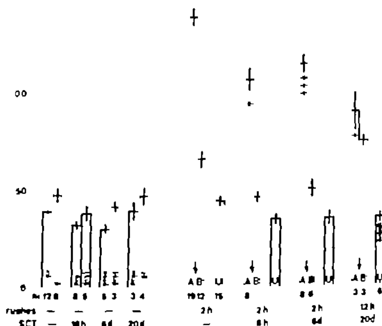


Fig. 2. The ACh cont nt of int ct or 12 h crushed rat sciatic nerve at arious times after spinal co transection (SCT) at the thoracic level V rious segments as indicated I Fig. 1 Arrow indicates site crush. Mean ± S.E. are given. d fferent from control A-segment at $p < 0.005$. different fr dist. uncrushed nerve of 20 days SCT rat at p 0.005

crush were dissected, as well as one or two 5 mm segments 10–20 mm distal t the sit of the crush (U + U) (Fig 1) 5 mm segments from comparative l vels of uncrushed nerves were dissected from which were not re-operated. In the physical exercise expt the A segment was 10 mm instead of 5 mm the nerves were dissected out 18 h afte the crush operatio (Fig 1). The animals were made unconsc by a blow on the head, the nerves were take out and mmediately put on an ice-cold plate and cooled pr to cutting. 2–6 nerve segme ts were pooled in each sample ACh w extracted according to MacIntosh Perry (1950) nd assayed on guine p g ileum preparation (Blaber and Cuthbert 1961). An isomet muscle transducer (Model 33-03-1693 Gould Inc. Instruments Syst. Div Cleveland, Ohio) was used regist r the contractions of the gut nd recordi g f the contractions was made on potentiometr recorder (Goertz Servogor S RE 541). Sch ld's four-po t assay technique (Schild 1942) was used fo calculating the results. An antihistamine (Mepyramine®) was added t the Krebs solutions used. Th activity i th samples was identif ed as ACh by inactivation with purified AChE (Worthington Bio chemical Corp., Freehold N.J) and by compl te inhibitio by tropine f the response of the gut standard and sample. The activity measured was thus ACh-like but will be referred to as ACh in the following.

For assays of *CAT and AChE* the sciatic nerves bilaterally were crushed both t the high level and 15 mm more distally Each site of crushing consisted f two crushes with 1–2 mm distance betwee the two crushes (Fig. 1). 12 h or 18 h (f exercise pts) later the rats were killed by decapitation, the nerves were dissected out, placed on a cooled plate and cut int 5 mm segment relative to the two sites of crushes. Comparative sections from uncrushed nerve were lso dissected and assayed for enzyme activities. CAT and AChE was assayed radiochemically in the same 5 mm segment according to the method of Tuček (1974), with some modifications.

Results

Decreased impulse activity

ACh. The level of ACh in the various nerve segments of control animals and SCT animals is shown in Fig. 2. In uncrushed nerves no significant changes in ACh-content were

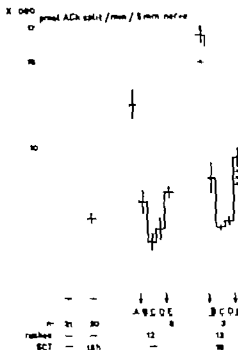


Fig. 3. AChE-activity in the sciatic nerve of control or 18 h spinal cord transected (SCT) rats. Arrows indicate site of crushes (see Fig. 1). Mean ± S.E. are given. Results from individual exp. * indicates different from control B segment, p 0.05. ** indicates different from control uncrushed nerve, p 0.01. *** indicates different from control A and segments, respectively p 0.005.

amounts of enzyme activity which was transported to the A-segment during 12 h (A minus uncrushed) increased after SCT as shown in Fig. 6.

Increased impulse activity

ACh In uncrushed nerves there was no marked difference in ACh content after physical training, as compared to control animals. In the A-section, however a 20% increase was seen in the exercised animals. The amount of ACh transported to the A-segment

$$\left(\text{A-prox} - \left(\text{B}\frac{\text{prox.}}{}\right)\right)$$

was increased from 117.5 pmol to 142 pmol, i.e. by 21 %. In the control material the "transportable" fraction of ACh

$$\left(\frac{\text{dist. uncrushed} \quad \text{U}}{\text{dist. uncrushed}}\right)$$

was 21 %. In the exercised animals, this calculation could not be done due to few observations and large variance (Fig. 7).

CAT No significant changes in enzyme activity was seen in uncrushed nerves after physical training and no difference was observed between the right and left nerves. In the 18 h crushed nerves the only segment which differed from control after training was the

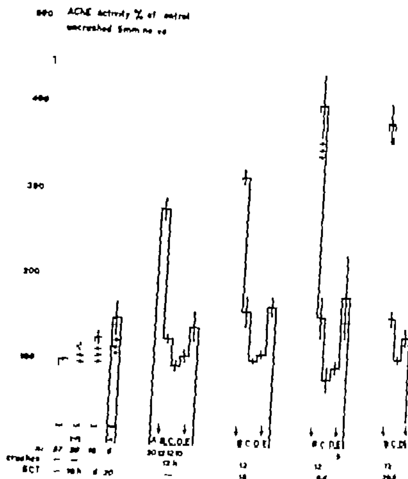


Fig. 4 AChE-activity in rat sciatic nerve at various times after spinal cord transection (SCT). Arrows indicate site of crushes (see Fig. 1). Mean ± S.E. are given. 100 = 4 882 ± 157 pmol of ACh split/min/mm nerve. indicates different from respective control nerve (uncrushed or 12 h crushed, segment A) at $p < 0.005$

was somewhat decreased at 6 days after SCT and somewhat increased by 20 days. The A-segment of 12 h crushed nerves contained significantly more CAT-activity in the 18 h SCT animals than in the controls. In the section between the two sites of crushes the CAT activity was redistributed, the middle piece (c) often containing the highest enzyme activity (Fig. 3)

AChE. In uncrushed nerves of the control group no significant difference in AChE activity between the various segments A–E could be noticed. Therefore all observations from uncrushed nerves were taken together as indicated in Fig. 4. In uncrushed nerves the AChE-activity gradually increased with time after SCT, reaching 150 % of control 20 days after SCT (Fig. 4). The A-segment of 12 h crushed nerves likewise showed an increase in enzyme activity with time after SCT, reaching maximum after 6 days. However already 18 h after SCT the A segment contained significantly increased AChE-activity when compared to the control group of that particular experimental occasion (Fig. 5). The C-segment contained the lowest enzyme level in all groups of animals and was about 5-15, 10, 35 and 30% lower than in uncrushed nerves of the respective groups (Fig. 4, 5). The

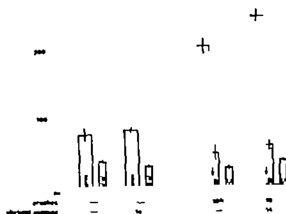


Fig. 7 The ACh-content of [illegible] or 18 h crushed sciatic [illegible] of rats, exercised for 14 days [illegible]. Mean + S.E. of the ACh [illegible] 5 or 10 mm nerve segments (indicated by different [illegible]) [illegible] to the crush level (see Fig. 1). [illegible] indicates different from [illegible] uncrushed nerve, distal [illegible], p 0.05 [illegible] indicates differ[ent] from control A-segment, [illegible] 0.05

The subcellular distribution of CAT and AChE in vivo

The subcellular intra-axonal distribution of the two enzymes appear to agree very well with that suggested for rabbit sciatic nerve (Tuček 1975). In rabbit sciatic nerve, AChE accumulated preferentially in the proximal 2 mm segment nearest a ligation while the maximal increase in CAT activity was observed in the segment 2–4 mm proximal to the ligation. This differential distribution may well be explained by the fact that AChE is bound in the axon to the sER which is transported at a rapid rate. This organelle thus occupies the space just above the ligation, displacing the axoplasm, containing the soluble CAT further away in a proximal direction. In the present study we dissected 5 mm segments, also proximal to the high crush levels, and have therefore most likely included both



Fig. 8 CAT-activity in uncrushed or 18 h crushed sciatic nerve of control and exercised rats. Arrows indicate sites of crushes. Mean [illegible] S.E. are given. Segments were dissected as shown in Fig. 1. [illegible] indicates different from control A [illegible] A [illegible] (taken together), p 0.05. [illegible] indicates different from control E segments.

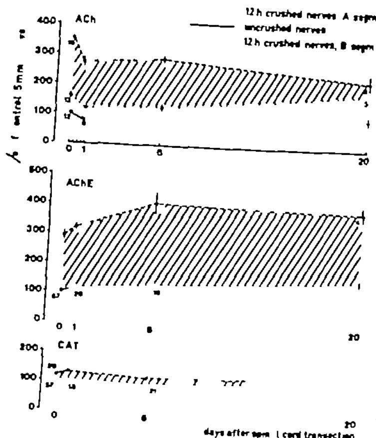


Fig. 6. Changes, caused by spinal cord transection, in the amount of proximo-distally transported substances in the rat sciatic nerve. The stripped areas show the amounts transported to the A segment, proximal to the high crush, during 12 h after the crush operation. Calculations performed as indicated in the text. Means ±S.E. are given, small figures show number of observations.

E-segment distal to the distal crush level (Fig. 8). However when segments A and A were taken together a small but significant decrease was observed after physical exercise.

AChE. No changes in enzyme activity was observed in any nerve segment after training.

Discussion

The methods used

The biological method to assay ACh used in this study has been discussed in a previous paper (Dahlström *et al* 1974 a). There is little doubt that the assayed activity really represented ACh. Support for this view is obtained by results from chemical identification of ACh in rat sciatic nerve (Ulin *et al* 1976) which gave similar results to ours concerning the amount of ACh per cm uncrushed sciatic nerve.

The enzyme methods used have been discussed by Tuček (1974). The activities of CAT and AChE in uncrushed rat sciatic nerve compare well with results of other investigators using other methods. Thus, CAT activity in rat sciatic nerve was earlier reported to be between 7–10 μmol of ACh formed/hr/g (Fonnum 1969, Saunders *et al* 1973). In the present study CAT-activity of uncrushed normal nerve was between 8–10 μmol of ACh formed/h/g, the weight of 5 mm nerve being about 5 mg. The AChE-activity of rat sciatic nerve was earlier found to be 56 μmol of ACh split/h/g (Kása *et al* 1973) while our own value in the present study was about 60 μmol of ACh split/h/g.

... rease the membrane-affinity of the CAT while the enzyme is mainly soluble during ... ionic conditions (Fonnum 1967 1968).

... in axonal transport by SCT

... this study we have tried to estimate the amount of the various substances that were ... ried with proximo-distal transport down the nerve during the 1 h crush period. For ... h this amount may be calculated according to the formula $ACh_A - ACh_B -$ ... $_{uncrushed}$ The ACh in segment A represents the content in uncrushed nerve plus the ... ount carried by axonal transport plus locally synthetized ACh, due to the axonal ... (cf Feldberg 1943). The ACh in segment B probably represents the amount in ... crushed prox. segment + the locally synthetized ACh (Dahlström et al. 1974 a). As can ... seen in Fig 2 the amount of ACh in segment B varied in the various groups of SCT ... The reason for this may be either changes in $ACh_{uncrushed}$ or changes in ... $Ch_{...}$ It is difficult to imagine that SCT can influence the local ACh synthesis ... llowing crushing. On the other hand, the $ACh_{uncrushed}$ was decreased in 18 h and 6 days ... CT animals (prox. segments, Fig. 2), when the ACh was below control levels, while at 20 ... ys after SCT the ACh in uncrushed proximal segments was again in the control range, ... it was the ACh_B of the 20 days SCT group Therefore, variations in ACh was probably ... due to variations in $ACh_{uncrushed}$

As can be seen in Fig. 6, the amount of ACh carried by axonal transport to the A ... segment *decreased* markedly with time after SCT The amount of transported AChE, on the other hand, calculated according to the formula $AChE_A - AChE_{uncrushed}$ (no local synthesis involved) was found to *increase* after SCT (Fig. 6, middle). A possible *decrease* in amounts of transported CAT may have occurred after SCT (Fig. 6, bottom)

It is very interesting that a decreased impulse activity has so pronounced effects on axonal transport of ACh and AChE as has been demonstrated in this study Previous studies have shown little or no effect of ... preganglionic denervation on intra-axonal transport of NA and amine granules in adrenergic neurons (Geffen and Rush 1968 Banks et al 1971) while an increased activity gave increases in amount of transported amine granules (Keen and McLean 1975).

Effects of physical exercise

After 14 days of daily physical exercise, which presumably caused net increase in the impulse activity of the motor neurons of the hindlimb, the amounts of ACh accumulated in the 1 cm long A-segment was clearly increased. The transported amounts appeared to be about 25 % higher than in the control group. The transportable fraction was about 5%

$$\left(\frac{\text{dist. uncrushed} \quad U}{\text{dist. uncrushed}}\right)$$

in the control group, but appeared less in the exercised group although no calculations could be performed due t few observations N clear changes were seen in the AChE expts., except that the proportion of proximodistally transported enzyme may hav been higher after exercise, as indicated by the pattern of redistribution in the segment between

the segment with high AChE-activity and high CAT-activity in our estimations on fast transport

In the 12 h double crush expts., both enzymes were redistributed within the segm isolated by the high and low crush sites, but in a very different manner suggesting a different subcellular distribution of the two enzymes

a) The *AChE* had moved towards both ends, leaving the middle segment (c) with the lowest activity The decrease from normal levels in this segment probably indicates the transportable fraction of AChE in the nerve (*cf* Lubińska and Niemierko 1971 Fonnum *et al* 1973 Tuček 1975) In all experimental groups in this study there was probably decrease below uncrushed levels in this segment, but the difference was statistically significant only for 6 days and 70 d SCT rats. In these two groups the decrease in the C-segment was 35 and 30%, respectively In most experimental groups the B-segment contained twice or more the AChE levels of the D-segment indicating that in the isolated rat sciatic nerve a larger proportion of AChE may move in the retrograde direction than in the proximo-distal direction. This is in contrast to the findings by Lubińska and Niemierko (1971) in the dog peroneal nerve and by Fonnum *et al* (1973) in the rabbit vagus and hypoglossal nerves, where the larger proportion of transportable AChE moved in the proximo-distal direction in isolated nerve segments. However Tuček (1975) found in rabbit sciatic nerve more AChE accumulating in the retrograde than in the anterograde direction One may wonder then why in our results the A-segment accumulated so much more esterase-activity than the E-segment because the reverse should be the case if retrograde transport was most prominent. Since two crushes were performed at each site of crush, contamination from A to B and from E to D may be ruled out One cause for the comparatively low enzyme level in segment E may be that the transportable fraction of AChE was exhausted at an early stage after crushing. The nerve length between the distal crush site and the motor endplates may be estimated to be only about 15 mm, and the transportable AChE-fraction of that 15 mm nerve would approximately correspond to the increase above uncrushed levels that was seen in the E-segment in *e.g* the 6 day SCT nerves (Fig. 4) The A segment, on the other hand, was continuous with the long proximal nerve and with the cell bodies, so there was no limit to the anterograde accumulation of AChE in this segment

b) The *CAT*-activity was in most cases higher in the middle C-segment of the isolated nerve than in the B and D segments. This may be due to the same displacement of axoplasm from the ends of a crushed fibre as occurred in the ends of rabbit sciatic nerves after ligation (Tuček 1975) Thus, it appears that in rat sciatic nerve the CAT is soluble and clearly separated in subcellular localization from AChE. In rabbit hypoglossal and vagal nerves the CAT appeared to be partly membrane affiliated since a decrease in the middle and increase towards both ends of an isolated nerve segment occurred (see Fig. 1 in Fonnum *et al* 1973) The results in the present study indicate that CAT is transported at a slow rate (a few mm/d) in agreement with the observation in rabbit sciatic nerve (Tuček 1975), but in contrast to the results from rabbit vagus and hypoglossal nerves (Fonnum *et al* 1973). These diverging results are difficult to explain, but possible reasons may be *e.g* variable osmolarity in various nerves, since it has been shown that hypotonic conditions

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the two crush sites (D-segment having slightly higher AChE activity than the B-segment The CAT was in main unchanged, but if the two segments above the high crush (A ... A) were calculated together a small but significant *decrease* was observed after exercis

The changes caused by increased impulse activity are comparatively small when compared to the changes induced by SCT It may be that other time intervals after onset of exercise could have induced more pronounced changes. Shorter intervals will be tested in a following study However an increase in the transported amount of ACh was present Since increased stimulation of a perikaryon is known to stimulate the protein synthesis (Hydén 1960), this observation was not unexpected. A decrease in the transport of CA was, however not in line with the increase in ACh transport

In the present study we have demonstrated that the impulse activity in motor neurons monitored by supra-spinal centres via descending axons, may regulate the synthesis and proximo-distal intra-axonal transport of what is probably ACh-storing organelles and of AChE and possibly also CAT in motor nerves. If alterations may occur in the axonal transport of these substances, it is likely that also transport of other substances, *e.g* hypothetical trophic substances (*cf* Gutmann 1964 Korr *et al* 1967 Ochs 1974) may occur following *e g* trauma of the spinal cord. Therefore, the atrophic changes observed in muscles of paralyzed individuals may be due not only to disuse of the limb but also to an altered delivery of trophic substances from the nerve to the muscles.

It may be speculated which descending systems that are most responsible for the control of the motor perikaryon with respect to the intra-axonal dynamics of ACh and the related enzymes. Unmyelinated axons may be involved since long-term proton irradiation of the spinal cord causing a severe demyelination of the white matter did not affect the transport of ACh AChE or CAT (Heiwall *et al* 1978) to any large degree The possibility that descending monoaminergic neurons are involved will be tested in a forthcoming paper.

S pported by the Swedish Medical Research Council (grants Nos. 14X 2207 04P-4173), by the Medical Faculty University of Got borg, by R ksföreningen for Traf k och Polioskadade by W & M Lundgrens V tenskapsfond, Greta and Ejnar Askers Stift lse nd by the S edish T bacco Company (grants Nos. 7310 and 7415).

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...e since then been published for many membrane systems (for references, see Ågren and ...quist 1976 a). Endogenous protein kinase was also demonstrated at the cell surface of both ...an glia and glioma cells in culture (Ågren and Ronquist 1974). This observation was ...ntly shown to be valid for also rat C-6 glioma cells (Schlaeger and Köhler 1976) as well as ...3T3 cells (Mastro and Rozengurt 1976). Furthermore Ehrlich cells, glia cells and ...l cells were able to phosphorylate exogenous acceptor proteins as phosvitin and histone ...gren and Ronquist 1974, Ronquist and Ågren 1974 b, Mastro and Rozengurt 1976). ...Ehrlich cells prelabeled *in vivo* with (^{32}P) orthophosphate either alone or in combina... ...with (^{14}C) glucose ...ere incubated with human glioma cells in culture resulting ...the recovery of labeled phosphorylserine and phosphorylthreonine from the glioma cells. ...me of the results have been published in a preliminary note (Ågren and Ronquist 1976 b). ...ese results support ... proposed model for possible interaction between cells (Ronquist and ...gren 1975). The model requires in addition ATP at the cell surface. An ATP formation at ...the cell surface has been shown to occur in both Ehrlich and glioma cells (Ågren and ...nquist 1969, Ågren *et al.* 1971).

Material and methods

...micals. All chemicals ...ere of analytical grade. (γ-^{32}P) ATP, (^{32}P) orthophosphate, D-glucose [^{14}C(U)] ...d L-leucine [^{14}C(U)] ...ere purchased from NEN Chemicals, GmbH, Frankfurt am Main, W. Germany. ...labeled ATP (sodium form), cyclic 3',5'-AMP and ADP ...ere obtained from Sigma Chemical Company ...Louis, Mo., USA.

...Preparation of Ehrlich cells. The Ehrlich ascites tumor cells were grown for 8–10 days in 5-weeks old ...ale ... mice were obtained from the Anticimex breeding farm, Norrviken, Stockholm. The Ehrlich ...cells were prelabeled *in vivo* ...ith 1 mCi of (^{32}P) orthophosphate either alone 24 h prior to sacrifice of the ...mice or in combination ...ith 100 μCi of ^{14}C-glucose. In the double-labeled experiments 100 μCi of ...^{14}C-glucose had also been injected intraperitoneally 52 h prior to sacrifice. In one series of experiments ...Ehrlich cells were prelabeled *in vivo* ...ith 10–20 μCi of ^{14}C-leucine 24 h before incubation ...ith unlabeled ...glioma cells. The labeled tumor cells were separated by centrifugation of ascitic fluid ...hich had been ...diluted about ... ten-fold ...ith ice-cold Krebs-Ringer bicarbonate medium, in order to diminish the ...tendency of cell agglutination. The cells were then washed twice in the Krebs-Ringer bicarbonate medium. ...Final washing ...as performed ...ith ... medium of 130 mM NaCl and 25 mM KCl just prior to incubation.

...Preparation of plasma membrane fraction from prelabeled Ehrlich cells

...Preparation of plasma membrane fraction from prelabeled Ehrlich cells was in accordance with the ...procedure given in ... previous work (Wernstedt *et al.* 1975) ...ith the exception that the EGTA-treatment ...during preparation ...as excluded. This ...as necessary since the observation ...as made that ... complete ...breakdown of the microsomal fraction occurred when the (^{32}P) orthophosphate injections had been ...given to the mice. This phenomenon ...as also seen when the radioactivity of (^{32}P) orthophosphate ...as ...lowered to 0.1 mCi per animal.

...Tissue culture conditions and preparation of glia and glioma cells for incubation experiments

...The cells were grown in Eagle's minimum essential medium ...ith 10 ... calf serum supplemented with ...penicillin, streptomycin and fungizone. All cultures ...ere grown at 37°C in ... 5 ... CO_2-humidified air ...(Ågren *et al.* 1971). After twofold washing at 37°C in the isotonic saline solution the cells were ...immediately used for incubation experiments.

...Incubation procedures

1. *Exogenous (γ-^{32}P) ATP added to glia and glioma cells.* The washed glia or glioma cells were incubated at 37°C with 10 nmoles of (γ-^{32}P) ATP to each plate (totally 40 plates ...ere used) in an isotonic medium ...containing 130 mM NaCl and 25 mM KCl, 35 mM Tris-HCl-buffer pH 7.5, 3.5 mM $MgCl_2$, 0.7 mM ...labeled NaH_2PO_4, 0.7 mM glutathione, reduced form (ordinary medium). In some experiments (Table

Acta physiol. scand 1978 103, 320–330

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Phosphoryl group transfer from Ehrlich cells prelabeled *in vivo* with (^{32}P)-orthophosphate and (^{14}C)-glucose to unlabeled glioma cells

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Received 19 January 1978

Abstract

ÅGREN G and G RONQUIST *Phosphoryl group transfer from Ehrlich cells prelabeled i vivo with (P)-orthophosphate and (C)-glucose to unlabeled glioma cells.* Acta physio scand 1978 *103* 320–330

A transfer of (^{32}P)-labeled phosphoryl groups has been demonstrated to occur between prelabel Ehrlich cell (do or cells) and glioma cells (acceptor cells). The Ehrlich cells were generally prelabele *i* *l* by an i traabdominal injection of (^{32}P)-orthophosphate 4 hours prior to sacrifice of the tumor beari g mice In some xperiments two i jections of (^{14}C)-glucose were given to the animals In additio to the i jection of (^{32}P)-orthophosphate prior to sacrifice The prelabeled Ehrlich cells ere mixed wi the glioma cells, that w re still attached t the culture plates, and incubation took place in vitro. Labele (^{32}P)-phosphoryl (^{14}C)-serine was isolated from the glioma cells as w ll as from the Ehrlich cells. The ... ratio of (^{32}P)-phosphoryl (^{14}C)-serine of the glioma cells (accept r cells) was significantly higher than for the Ehrlich cells (donor cells). This was indicativ of nteractive mechanism between the cel partly due t binding of membrane fragments from th Ehrlich cells on to the surface of the glioma cel and partly due t phosph ryl group transfer betwee the two types of cells. Interaction experimen with a plasma membrane fraction prepared from the prelabeled Ehrlich cells is à vis the glioma cel clearly showed that the phosphoryl group transfer mechanism between the cells was dominating a only mino part of the nteraction was due t adhesion. This transphosphorylation reaction represe a new type of interaction between cells. It was not influenced by ther adenosine 3 ,5 -monophospha or adenosine 5 -diphosphat

A membrane bound protein kinase which phosphorylates endogenous membrane protei was first found in Ehrlich ascites tumor cells (Ågren and Ronquist 1970, Ronquist an Ågren 1970 Ågren and Ronquist 1971) Using exogenous (γ-^{32}P)ATP as substrate, it wa apparent that the reaction took place at the outer surface of the intact Ehrlich cells an resulted in phosphorylation of protein-bound seryl and threonyl-residues of the accept protein(s) (Ågren and Ronquist 1970 Ronquist and Ågren 1970, Ågren and Ronquist 197 Ronquist and Ågren 1974 a). Protein kinase-catalyzed membrane phosphorylation eith involving the endogenous protein kinase and extrinsic acceptor protein or an extrins enzyme and the endogenous acceptor or where both acceptor and enzyme are endogeno

[illegible] I (^{32}P) phosphoryl group transfer from (γ-^{32}P) ATP into cell surface of glia and glioma cells. Average values from 2 expts. are given in picomoles per 1 × 10⁶ cells.

	Glia cells	Glioma cells
phosphorylserine residue	1.49	0.33
phosphorylthreonine residue	0.37	0.05

[illegible] endogenous phosphoryl group acceptor protein(s) seemed to be less labeled in glioma cells [than] in glia. This is congruous with the earlier findings of less protein kinase activity at the [glio]ma cell surface compared with glia in the presence of exogenous acceptor protein [(Ågren] and Ronquist 1974). On the contrary Rozengurt and Heppel (1975) compared [the] normal and virus-transformed malignant pair of Swiss 3T3 cells and claimed that the [malig]nant cells were phosphorylated by exogenous ATP while the normal cells were not. [Fi]g. 1 A-1 E illustrate the Ehrlich cell behaviour on the plates during the incubation and [wash]ing procedures. Fig. 1 A illustrates washed glioma cells attached to the plates immedia[tely] before incubation. Fig. 1 B shows Ehrlich cells and glioma cells during incubation. [It is] evident that the Ehrlich cells cover the glioma cells completely. Fig. 1 C shows that [the] Ehrlich cells are still present after the first washing. The number of Ehrlich cells is even [mo]re reduced after the second washing (Fig. 1 D) while no Ehrlich cells are visible after the [thi]rd washing (Fig. 1 E).

As is given in Table II it was possible to isolate measurable amounts of labeled phos[ph]orylserine and phosphorylthreonine in four consecutive experiments on different [occa]sions from the glioma cells after incubation with Ehrlich cells prelabeled *in vitro* with [(^{32}P)] orthophosphate. There might exist different modes for the quantitative determination [of] phosphoryl groups transferred from donor to acceptor cells. One is based on the assump[tio]n that about 1% of the ATP content of the Ehrlich cells is available at the cell surface [(]Ågren and Ronquist 1969, Wernstedt *et al.* 1975) and that the (^{32}P) orthophosphate has [co]me into equilibrium with the different phosphate pools of the Ehrlich cell. On this basis [th]e mean values for phosphoryl group transfer from Ehrlich to glioma cells were 0.37 [pic]omoles of labeled phosphorylserine per 1 × 10⁶ glioma cells. The corresponding figure for [p]hosphorylthreonine was 0.060 picomoles (Table II). Correction has not been made for [de]stroyed phosphorylserine and phosphorylthreonine during acid hydrolysis. It is also seen [i]n Table II that ADP or cyclic AMP did not influence the transphosphorylation reaction. Also, cyclic AMP has not shown any stimulating activity on the membrane protein kinase [o]f Ehrlich cells and glioma cells in earlier experiments with exogenous (γ-^{32}P) ATP as the [ph]osphoryl group donor and endogenous acceptors (Ronquist and Ågren 1974, Ågren and Ronquist 1976, Ronquist *et al.* 1977). A modified Eagle's medium (Ca²⁺-free) was used in [so]me experiments instead of the buffered saline solution. The labeled phosphoryl group [tra]nsfer into Ser-^{32}P under otherwise identical conditions was reduced by about 50%. One [pos]sible explanation for that might be that the (γ-^{32}P)ATP at the cell surface of the donor [c]ells is less available for the transphosphorylation reaction in the presence of albumin in the [inc]ubation medium (*cf.* Fig. 3 in Wernstedt *et al.* 1975).

[I]n an experimental system comprising phosphoryl group transfer between cells it is

II) ADP and 3 5 AMP were included respectively Furthermore in another experiment the isotonic and K solution buffered with TrisHCl was e changed for Eagle s medium lacking Ca^{2+} Total incubation volume was 3 ml Incubation proceeded for 1 min and was terminated by pouring off the medium from the plates containing the ttached cells. The cells were further washed thrice in the NaCl-KCl-medium prior to precipitation with 3 ml of warm 3 per cent sulfosalicylic acid (SSA) and in the acid medium for 3 min. The SSA-treatment was repeated once. The destroyed cells were kept wet in a minimal amount of SSA until removal of the cells from the plate (Ronquist et al. 1970).

2. *(^{32}P) orthophosphate pre-labeled Ehrlich cells incubated ith glioma cells.* After the final washing prelabeled Ehrlich cell w re immediately suspended in the above-mentioned incubation medium lacking (γ-^{32}P) ATP The cytocrit was adjusted to give a total of bout 12 million cells per 3 ml cell suspension. suspension was kept i ice-w ter and unde oxygen (96)-carbondioxide (4) tmosphere until Each plate of the glioma cells (about 6 millions per plate) were incubated after the final washing 3 ml of the Ehrlich cell suspension pretreated in a water bath at 37°C immediately before the incubation experiment. Incubation proceeded for 3 min and wa terminated by pouring off the Ehrlich cells the plate still containi g the attached glioma cells. The incubated glioma cells ere ashed and treated with SSA as described bove

3 *^{14}C-glucose- and (^{32}P) orthophosphat double-labeled Ehrlich cells incubated ith glioma cells* double-labeled Ehrlich cells were incubated with glioma cells exactly as described above. The treatment of the incubated glioma cells was also in accordance with the abovementioned description. The double labeled Ehrlich cells were also washed thrice in the isotonic saline solution after incubation The cells were then precipitated in 5 per cent trichloroacetic acid (TCA). The denatured Ehrlich cells washed once n 5 per ce t TCA containing an excess of unlabeled orthophosphate and ATP

4 *C-leucine-prelabeled Ehrlich c lls incubated ith glioma cells.* I order to rule out possible transfer of labeled membrane fragments from the prelabeled Ehrlich cells on to the glioma cell surfaces following controls were carried out The Ehrlich cells were prelabeled *in vivo* with 10–20 µCi of leucine 24 h before the incubation with the unlabeled glioma cells. The incubation and washing procedures were identical with those described above with one exception After the incubation the glioma cell plates were w shed three t mes w th the isotonic salt solution followed by three washings with hot (90 C) SSA. As is seen in T ble III the first and second washi gs from the incubated glioma cells contained radioactive material contrary to the third washing.

5 *Plasma membrane fraction prepared f m (^{32}P) prelabeled Ehrlich cell incubated ith glioma cells* A plasma membrane fraction was prepared from 3 6 10^9 Ehrlich cells prelabeled *in vivo* ith (^{32}P) orthophosphate by i jection of 0.1 mC (^{32}P) orthophosphate n each a imal. The labeled membrane fraction corresponded t about 45 mg protein. The membrane material was prepared just prior to incubation and maintained t 0° unt l incubation The material was equally distributed i to 39 plates containing washed glioma cells. Incubation proceeded for 3 min at 37°C nd was terminated by pouring off the labeled plasma membrane fraction. Washi gs f the incubated glioma cells were in accordance with procedure given above a d the glioma cells and membrane material from the Ehrlich cells ere treated with hot 3 SSA as given above.

Acid hydrolysis f cell material and isolation of labeled phosphoryl serine and phosphorylthreonine

After incubatio the acid-treated Ehrlich cells nd plasma membrane fraction were concentrated according t Schneider (1945) and partially hydrolyzed following the method of Lipmann (1933). The small amount of glioma cell material did not permit Schneider proced re and as therefore directly hydrolyzed given above. Labeled phosphoryiseri e and phosphorylthreonine were isolated by Dowex 50 column chromatography as previously described (Ågren a d Ronquist 1970, Ronquist and Ågren 1970).

Radioactivity was determined i a Nuclear Chicago Sc tillator Counter and ^{32}P radioactivity determined by measuring the Cherenkov radiation I the experiments w th double-isotope labeling ^{32}P-determination was performed n connectio with the experiment while the ^{14}C-determination made after bout 2 months in orde to facil tate the discriminative measuring.

Cell counts were done lectronically Linson counter 411 celloscope

Results

Table I demonstrates the presence of endogenous acceptor proteins at the cell surface both glia and glioma cells using (γ-^{32}P) ATP as exogenous phosphoryl group donor T

[illegible] (^{32}P)-phosphoryl group transfer from donor cells into cell surface of acceptor cells under various conditions. The values are given in picomoles per 1×10^{6} cells. ADP concentration was 0.73 mM and 3′5′cyclic AMP concentration was 1×10^{-4} M. Calcium ions were excluded from the Eagle's medium. Range of values for phosphoryl serine and phosphorylthreonine residues from 4 expts. are given in brackets

	Ordinary medium	ADP in medium[a]	3′5′ cyclic AMP in medium[b]	Eagle modified medium[a]
Phosphoryl-[illegible] residues	0.37 (0.25–0.54)	0.33	0.32	0.16
Phosphoryl-[illegible] residues	0.06 (0.02–0.11)	0.07	0.06	—

[a] [illegible] expt.
[b] Mean value of 2 expts.

to exclude the possibility of transfer of peptides and membrane fragments. [illegible] fact, the occurrence of peptides and proteins at the outer surface has been reported [illegible] Gates *et al.* 1974, Rozengurt and Heppel 1975). Therefore, experiments were [illegible] out [illegible] Ehrlich cells prelabeled *in vitro* with [illegible]C leucine. Such cells were incubated [illegible] glioma cells (Table III). The [illegible]C-leucine content of Ehrlich cells after incubation [illegible] the glioma cells (3 washings with isotonic salt solution are included) was 122 512 cpm. [illegible] also seen in the Table, the "blind" washings (empty plates incubated with Ehrlich [illegible] with [illegible]C-leucine) with hot 3% SSA did not contain any radioactivity while [illegible] remaining washed and SSA-treated glioma cells contained 64 cpm, *i.e.* 0.04 per cent [illegible] the total activity of the Ehrlich cells. Since the detached, incubated glioma cells always [illegible] a small [illegible]C-activity the possibility of a transfer of labeled fragments from the [illegible] cells could not be completely excluded. Hence Ehrlich cells were also labeled on two [illegible] occasions with (^{14}C)-glucose as well as with (^{32}P)-orthophosphate. The double-[illegible] (^{14}C)-serine (^{32}P)- and (^{14}C)-threonine (^{32}P)-phosphoryl residues were isolated in [illegible] way both from the donor cells (Ehrlich cells) and from the acceptor cells (glioma [illegible]). The amount of labeled residues were calculated by measuring (^{14}C)- and (^{32}P)-

[illegible] III ^{14}C-radioactivity of prelabeled Ehrlich cells, the three [illegible] washings and glioma cells after incubation. The Ehrlich cells were prelabeled *in vitro* 24 h before incubation with the glioma cells [illegible] 10–20 μCi of ^{14}C-leucine. The [illegible] labeled Ehrlich cells [illegible] incubated with the glioma cells as given in text. The figures denote total counts per min.

Total radioactivity (cpm)

	Ehrlich cell suspension after incubation	1st washing with 3	1st washing SSA	2nd washing with 3	2nd washing SSA	3rd washing with 3	3rd washing SSA	Detached glioma cells after the washings
[illegible] containing [illegible] cells	123 512	4 524		1 416		0		64
[illegible] plates	150 304	1 416		0		0		0

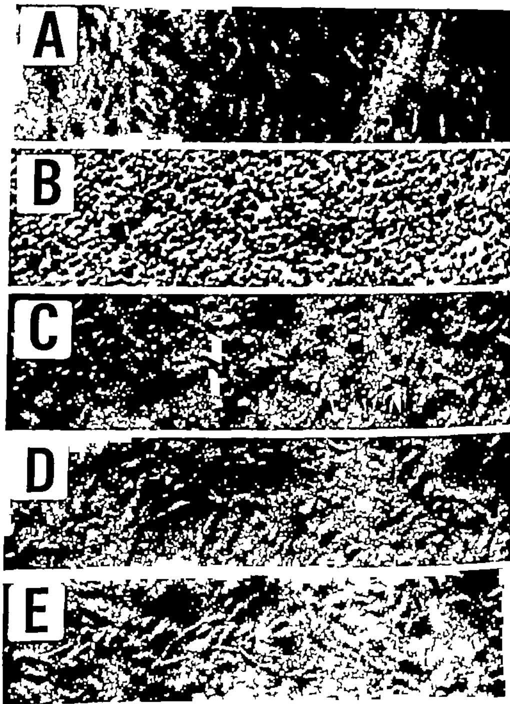


Fig. 1. *A*. Photograph of washed glioma cells attached to the plates immediat ly before incubation. × *B*. Prelabeled Ehrlich cells (1 10^{10} cells) cove completely the glioma cells (1 10^{4} cells). Photograph tak during incubation. × 250. *C*. Glioma cells ft completion of incubation. The Ehrlich cells have be separated from the glioma cell and they have been washed once w th the isotonic salt sol tion. F Ehrlich cells are st ll seen among the glioma cells × 250. *D*. The mber f Ehrlich cells is even mo reduced after the second washing × 250. *E*. N Ehrlich cells re visible aft the third washing of t glioma cells. × 250.

[...]LE II (³²P) phosphoryl group transfer from donor cells into cell surface of acceptor cells under various conditions. The values are given in picomoles per 1 × 10⁶ cells. ADP concentration was 0.73 mM and 3'5'cyclic AMP concentration was 1 × 10⁻⁶ M. Calcium ions were excluded from the Eagle's medium. Range of values for phosphoryl serine and phosphorylthreonine residues from 4 expts. are given in brackets.

	Ordinary medium	ADP in medium[a]	3'5' cyclic AMP in medium[b]	Eagle's modified medium[a]
[...]P) phosphoryl-[...]ine residues	0.37 (0.25–0.54)	0.35	0.32	0.16
[...]P)phosphoryl-[...]reonine residues	0.06 (0.02–0.11)	0.07	0.06	—

[a] [...]ingle expt.
[b] [...]ean value of 2 expts.

[...]essary to exclude the possibility of transfer of peptides and membrane fragments. [...] fact, the occurrence of peptides and proteins at the outer surface has been reported [...] *e.g.* Gates *et al.* 1974, Rozengurt and Heppel 1975). Therefore, experiments were [...]ried out with Ehrlich cells prelabeled *in vivo* with ¹⁴C-leucine. Such cells were incubated [...]h glioma cells (Table III). The ¹⁴C-leucine content of Ehrlich cells after incubation [...]h the glioma cells (3 washings with isotonic salt solution are included) was 122 512 cpm. [...] is also seen in the Table, the "blind" washings (empty plates incubated with Ehrlich [...]s prelabeled with ¹⁴C-leucine) with hot 3% SSA did not contain any radioactivity while [...] remaining washed and SSA-treated glioma cells contained 64 cpm, *i.e.* 0.04 per cent [...]he total activity of the Ehrlich cells. Since the detached, incubated glioma cells always [...]tained a small ¹⁴C-activity the possibility of a transfer of labeled fragments from the [...]rlich cells could not be completely excluded. Hence Ehrlich cells were also labeled on two [...]ferent occasions with (¹⁴C)-glucose as well as with (³²P)-orthophosphate. The double [...]eled (¹⁴C)-serine (³²P)- and (¹⁴C)-threonine (³²P)-phosphoryl residues were isolated in [...] usual way both from the donor cells (Ehrlich cells) and from the acceptor cells (glioma [...]s). The amount of labeled residues were calculated by measuring (¹⁴C)- and (³²P)-

[...]LE III ¹⁴C-radioactivity of prelabeled Ehrlich cells, the three washings and glioma cells after incubation. The Ehrlich cells were prelabeled *in vivo* 24 h before incubation with the glioma cells with 10–20 µCi of ¹⁴C-leucine. The washed labeled Ehrlich cells were incubated with the glioma cells as given in text. The figures denote total counts per min.

	Total radioactivity (cpm)							
	Ehrlich cell suspension after incubation	1st washing with 3%	1st washing SSA	2nd washing with 3%	2nd washing SSA	3rd washing with 3%	3rd washing SSA	Detached glioma cells after the washings
[...]) containing [...]ma cells	122 512	4 524		1 416		0		64
[...]pty plates	150 304	1 416		0		0		0

TABLE IV Radioactivity of double labeled (^{32}P)-phosphoryl (^{14}C)-serine residues in donor cells and acceptor cells. Ehrlich cells were prelabeled *in vivo* twice 52 h and 24 h before incubation. At first and second prelabeling 100 μCi of ^{14}C-glucose were administered intraperitoneally 1 of (^{32}P)-orthophosphate was also included in the second injection. Figures denote the respective (^{32}P)-phosphoryl and ^{14}C-radioactivity in counts per min per 1 10^{8} donor and acceptor cells respectively

	(^{32}P) phosphoryl radioactivity	^{14}C radioactivity	^{32}P/^{14}C ratio
Donor cells (Ehrlich cells)	181 083	18 973	9 6
Acceptor cells (glioma cells)	1 129	67	16.9

radioactivity The results are given in Table IV It is seen that labeled phosphorylserine residues were recovered from the glioma cells amounting to 0 62% of the total Ser ^{32}P of the donor cells calculated on P measurements. If instead this measurement of the recovered labeled phosphorylserine residues was made on ^{14}C-counting of phosphoryl-(^{14}C) serine the corresponding figure amounted to only 0.35%. Thus, there is a discrepancy between the (^{32}P)-phosphoryl and ^{14}C-countings of the recovered phosphorylserine residues of glioma cells indicating a changed stoichiometric relationship between the (^{32}P)-phosphoryl-(^{14}C) serine residues of the donor cells and (^{32}P)-phosphoryl (^{14}C)-serine residues of the acceptor cells favouring the view that the increased ^{32}P/^{14}C ratio of the serine residue of the acceptor cells is due to a transphosphorylation from the donor cells into the acceptor cells

Table V illustrates that 4 1 per cent of the total (^{32}P)-radioactivity of the Ehrlich cells are recovered in the plasma membrane fraction Furthermore, 0 11 per cent of the total activity are recovered in the (^{32}P)-phosphoryl residues after partial hydrolysis of this plasma membrane fraction. It is also seen in the table that the total cell count corresponds to 1 050 mg protein. 4.3 per cent of this amount is recovered in the plasma membrane fraction. This figure is close to the percentage value of radioactivity distribution. It is noteworthy since the radioactivity and protein measurements were made on different cell preparations. The set of 6 values given in Table VI has been obtained on 6 different expts. performed during a two years period A mean value of 0 019 per cent of the total (^{32}P)-radioactivity of the Ehrlich cells is recovered in the (^{32}P)-phosphorylserine residues of the glioma cells.

TABLE V Distribution of (^{32}P)-radioactivity and protein in Ehrlich cells and subfractions from the cells. The Ehrlich cells were prelabeled *in vivo* as given in text

	(^{32}P) radioactivity in cpm. (millions)	Per cent of total radioactivity	Protein content (mg)
Ehrlich cells (3.6 10^{9})	171 1	100	1 050
Plasma membrane fraction	7 1	4 1	45
Plasma membrane fraction purified according to Schneider	2.7	1 6	
(^{32}P)-phosphorylserine residue isolated after partial hydrolysis	0.18	0.11	

TABLE VI. Relationship between total counts in Ehrlich cells and (^{32}P)-phosphorylserine residues of glioma cells

Total counts in Ehrlich cells (cpm in millions)	Total counts of (^{32}P)-phosphorylserine residues of glioma cells (cpm)	Per cent recovery of radioactivity in (^{32}P)-phosphorylserine residues of glioma cells
6.9	756	0.011
5.6	1 364	0.025
16.3	2 238	0.014
16.0	3 870	0.024
9.5	2 997	0.032
14.5	1 463	0.010

Mean per cent value from 6 different expts. = 0.019

It is known that cell membrane fragments can bind to the surface of intact cells under certain conditions (Öbrink *et al.* 1977). Therefore it was necessary to exclude the possibility of a binding between membrane fragments from the Ehrlich cells and the surface of the glioma cells. A plasma membrane fraction was prepared from 3.6 $\times$ 10^{8} Ehrlich cells prelabeled *in vivo* with (^{32}P)-orthophosphate. The radioactive membrane fraction, corresponding to 45 mg protein, was incubated with the glioma cells. An extremely small amount of (^{32}P)-phosphorylserine residues barely detectable with radiometric methods was recovered from the incubated glioma cells (Table VII). The figure obtained from the glioma cells only amounted to 1.9 per cent of the expected value calculated on an ordinary interaction between the prelabeled Ehrlich cells and the glioma cells (see Tables V and VI).

Discussion

The interaction between two types of cells as demonstrated in the present paper requires basically some prerequisits. They include the presence of protein kinase and an endogenous phosphoryl group acceptor at the cell surface. Furthermore, ATP is necessary as the

TABLE VII. Radioactive recovery of (^{32}P)-phosphorylserine residues from glioma cells after incubation with radioactive plasma membrane fraction from prelabeled Ehrlich cells. Relationship to expected (calculated) value for total radioactivity of (^{32}P)-phosphorylserine residues from glioma cells.

	Radioactivity in counts per min
Total radioactivity of plasma membrane fraction prepared from 3.6 $\times$ 10^{8} Ehrlich cells	933 296
Total (^{32}P)-radioactivity recovery of phosphorylserine residues from glioma cells	81
Expected (calculated) value for total radioactivity of (^{32}P)-phosphorylserine residues from glioma cells	4 318[a]

[a] Figure obtained by dividing 933 296 with factor 216.15
[b] Factor obtained by multiplying 37.27 (4.1 $\times$ 0.11 see Table V) with 5.8 (0.11 (Table V) : 0.019 (Table VI)).

phosphoryl group donor. The presence of protein kinase at the cell surface has been demonstrated on different cells in our laboratory and confirmed by others (Mastro and Rozengurt 1976, Schlaeger and Köhler 1976). The endogenous acceptor is present at the cell surface of glia as well as glioma cells (Table I) although the latter cells display a lower degree of phosphorylation compared with the former cells contrary to findings on another pair of normal and malignant cells (Rozengurt and Heppel 1975). The existence of a ATP forming capacity at the surface of Ehrlich cells has been discussed (Ågren and Ronquist 1969, Ågren and Ronquist 1975, Wernstedt *et al.* 1975). However one might argue that (^{32}P)-orthophosphate may permeate from the donor cells into the medium and the medium into the acceptor cells. Labeled orthophosphate can then be incorporated in the terminal position of ATP by glycolysis in the latter cells and thereafter take part in phosphorylation reactions of these cells. In order to nullify the phosphate gradient into the incubation medium all experiments were carried out in the presence of $1 \cdot 10^{-4}$ M unlabeled orthophosphate. Furthermore, even in the absence of equilibrated gradients, the phosphate transport through the plasma membrane is slow (Ågren and Ronquist 1969). Moreover when exchanging the exogenous (γ-^{32}P) ATP for (^{32}P) orthophosphate in the incubation medium, the labeling of the endogenous peptide of the surface membrane of Ehrlich cells was negligible even after a 5 min incubation period (Ågren and Ronquist 1971). Therefore, it seems unlikely that the phosphoryl group transfer studied was caused by labeled orthophosphate transported from the Ehrlich cells into the glioma cells.

Another possibility would be the binding of possible labeled membrane fragments from the Ehrlich cells on to the surface of glioma cells. Such an adhesive specificity between some other celltypes has been claimed to occur (Öbrink *et al.* 1977). However, this binding was confined to one cell type and certain organells from these cells. Experiments performed with ^{14}C-leucine-prelabeled Ehrlich cells could favour such a hypothesis since a slight amount of ^{14}C-labelling was recovered in the glioma cells after incubation with the Ehrlich cells (Table III). Experiments with Ehrlich cells, double labeled with ^{14}C-glucose and (^{32}P)-orthophosphate again seemed to indicate a certain small degree of binding, since the recovered (^{32}P)-phosphorylserine residue fraction not only contained (^{32}P)-radioactivity but also some ^{14}C-radioactivity (Table IV). The ^{32}P/^{14}C ratio was however different for the acceptor cells compared with the donor cells suggesting in addition a phosphoryl group transfer. It should however be kept in mind that the phosphorylserine residue fraction is heterogeneous (Ågren *et al.* 1962, Ronquist and Ågren 1974 a) containing several non-essential amino acids as well as hexoseamine. These compounds might contain a high-specific labeling of ^{14}C-carbon while lacking (^{32}P)-labeling. Thus, a minor component of binding of membrane fragments from the Ehrlich cells onto the surface of glioma cells cannot be ruled out. A comparison is difficult to perform stoichiometrically since one molecule of *e.g.* hexoseamine can be labeled maximally on all six carbone atoms while a molecule of phosphorylserine can only maximally be ^{32}P labeled in one position. Hence, it was not possible to estimate on a quantitative basis how much of the recovered labeling in the glioma cells was due to phosphoryl group transfer and binding of membrane fragments, respectively.

A promising step forward along this line seemed to be the incubation experiments with

the plasma membrane fraction prepared from prelabeled Ehrlich cells. Such a plasma membrane preparation contained about 4 per cent of the total (^{32}P)-radioactivity of the Ehrlich cells. The preparation of the plasma membrane fraction included several purification steps and it seemed likely that loosely bound low molecular compounds and cofactors including ATP had been removed. Therefore, since apparently no phosphoryl group transfer was possible, conditions were available for study of a possible binding reaction between the plasma membrane fraction containing (^{32}P)-phosphoryserine and glioma cells. It was clearly demonstrated that only 2% of the (^{32}P)-labelling of the glioma cells could be due to binding of membrane fragments onto the surface of glioma cells while the majority of the ^{32}P-labeling most probably was caused by a phosphoryl group transfer.

In conclusion we have demonstrated a new type of interaction between cells involving a phosphoryl group transfer. The physiological significance of such a reaction is at present not clear but one possibility might be a regulatory phosphorylation at the cell surface (cf. ref. Ronquist et al. 1977). This also implies a dephosphorylation reaction at the cell surface. Such a reaction has recently been found at the surface of intact Ehrlich cells as well as in the plasma membrane fraction (unpublished results).

This investigation was supported by a grant from the Swedish Medical Research Council (Project No. 13X-04224-14C). We thank Drs J. Pontén and B. Westermark for kindly providing us with glia and glioma cells. We are also indebted to Mr B. Eklund and M. C. Wernstedt for their technical assistance.

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Functional recovery following cross-reinnervation of antagonistic forelimb muscles in rats

By

AVIS H. COHEN

[illegible]veral early studies in mammals (Barron 1934 and Sperry 1947) indicated the return of [illegible]rmal function *following* cross-innervation of antagonistic muscles. Mammalian muscle [illegible] generally be innervated by only one nerve fiber. If a second axon does innervate a [illegible]uscle fiber both sets of neuromuscular junctions are functional (Frank *et al.* 1975). Thus, [illegible] in mammals once a muscle is cross-reinnervated normal function can only be restored by [illegible] central reorganization. The results of Sperry (1942, 1947) denied that such reorganization could occur. The present study in rats reexamines this problem.

Single pairs of antagonistic forelimb muscles were cross-innervated and their subsequent electromyographic (EMG) pattern examined. Unlike Sperry's rats the animals were left otherwise intact. Sperry had removed the remaining limb musculature, the paw, the contralateral limb and the tail. Although the majority of animals in the present study confirmed Sperry's findings, some did display alteration in the activity of the crossed muscle nerve.

[illegible] 40–60 g [illegible] albino rats were anaesthetized with sodium pentobarbital (50 mg/kg) and the nerves to the medial head of the triceps (MhT), an extensor, and the long head of the biceps (LhB), a flexor were dissected to the point of entry into the muscles. A [illegible]ery fine nylon monofilament suture (Ethicon, 9-0) was tied around the nerve and [illegible] small piece of underlying muscle tissue, and reattached to the antagonistic muscle at the region from which the original nerve had been removed. Sham operated animals ([illegible] = 2) were treated in the same [illegible]y but the nerves were reattached to their original muscles.

After 4–6 months electromyography [illegible] used during locomotion to test the function of the cross-innervated MhT muscles. The long head of the triceps was the control for the crossed MhT. During [illegible] and trot, these two heads of the triceps fire at the same time. All tests included bipolar EMG recordings from the normal long head of the triceps (LhT) and operated medial head of the triceps (MhT) (see Cohen and Gans 1975 for technique). Coincident bursts [illegible] the MhT was functioning normally. Animals were run at least twice [illegible] separate electrode insertions.

In 10 animals innervation to the reinnervated MhT's was evaluated by determining the twitch tension produced by stimulating the flexor and extensor nerves, including 2 [illegible] MhT [illegible] had exhibited only extensor-type activity (see below). Under urethane anaesthesia (1.[illegible] g/kg) all the forelimb musculature was removed except the MhT and LhB. The proximal extensions of the flexor and extensor nerves were exposed and cut. The humerus [illegible] fixed. The MhT [illegible] placed at an anatomically appropriate angle, with its tendon attached to an isometric strain gauge and the muscle set to produce maximal twitch tension. The nerves were mounted on bipolar stimulating electrodes and the muscles bathed in paraffin oil (38°–39°).

Tension measurements revealed that in the MhT's of 7 animals tested there was flexor and extensor innervation. In 3 animals *only* the foreign flexor innervation produced tension (Table I, see Legend).

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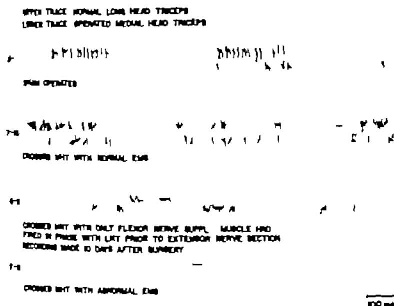


Fig. 1. EMG recordings of 4 animals. The upper trace of each pair is the normal long head of the triceps and the bottom trace is the operated medial head of the triceps. The number above each pair of traces refers to the animal. Note, in animal 7-8, although there was no measurable tension produced by the triceps (Table I) there was recordable extensor-type EMG-activity. Thus, in view of the large tension generated by the biceps nerve in MNT of 7 16 it is unlikely that the nerve was producing undetected flexor-type activity.

changes may be due to a modification in the "central program for locomotion or to the establishment of a new program. It might, however simply reflect a tonic suppression of the flexor motoneurons centrally or by peripheral feedback from normal or abnormally innervated sensory receptors. If further experiments can corroborate these results the view that no central reorganization can occur must be abandoned.

This work formed part of a doctoral dissertation submitted to the Division of Biological Sciences Cornell University. It was completed under the direction of Drs R. R. Capranica and Carl Gans. The mechanical properties of the crossed muscles were investigated in the laboratory of V. R. Edgerton, U.C.L.A. with the aid of Dr Edgerton, R. Rhombart and D. Simpson. Comments by Dr S. Grillner were extremely helpful in the production of this manuscript.

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TABLE I Twitch Tensions of the operated MhT's generated by stimulation of the foreign or original nerve. f/e = flexor/extensor-type EMG e = extensor-type EMG There are 3 possible explanations f... the extensor bursts in 6–6, 7 3 and 7–8 1) The force transducer could not measure tension belo... 1 g. Thus, if less than 1 g of tension was produced by extensor innervation, it ... ould be undetec... 2) the extensor nerve was injured duri g the dissection 3) the flexor innervation was active dur... the extensor phase of the step cycle.

Animal	Cond tion EMG pattern	Tension (g) Nerve stimulated	
		Triceps	Biceps
6–3	crossed f/e	4 9	8.9
6–6	crossed f/e	0	4.3
6–13	crossed f/e	7 6	6..
7 3	crossed f/e	0	9.3
7 5	crossed f/e	13 9	15.5
7–8	crossed f/e	0	15 9
7 9	crossed f/e	12.2	6.4
7 11	crossed f/e	11.0	3 1
6–9	crossed e	10.7	3.5
7 16	crossed e	10.7	12..
7 17	sham	15.3	—

Flexor/extensor type EMG In 15 of 19 animals the EMG pattern consisted of two burs... of activity per step cycle one in-phase with the control LhT (extensor-type burst), and one out-of-phase with the LhT (flexor-type burst) Presumably these bursts were produced by the extensor and flexor nerves, as the timing of these bursts was essentially identical to that seen in normal animals (Cohen and Gans 1975). The 15 animals included 3 with no measurable tension associated with the extensor nerve (cf 7–8 Fig. 1 Table I)

Extensor type EMG In the remaining 4 animals the cross-reinnervated MhT's exhibited only *one* burst per stride (Fig. 1 7–16), coinciding with the activity of the LhT The muscle was silent during the flexor phase of the step cycle The presence of functional flexor innervation to the MhT's of two of these 4 animals was confirmed by stimulating flexor and extensor nerves. Both produced substantial tensions (Table I) With the techniques used it was impossible to determine whether the flexor nerve was contributing to the EMG activity

Subsequently in a third an mal from this group, under anaesthesia the extensor innervation to the MhT was cut as far proximal to the site of crossing as possible This left only the muscle s flexor innervation intact. 5 days later the activity of the MhT was reexamined. During th s and another test 10 days later the MhT exhibited only a strong flexor burst (F g. 1 6–11). Six months later the activity was still pure flexor-type. The flexor EMG activity could have been a product of newly sprouted flexor nerve fibers, but it is unlikely that this would be major factor just 5 days after nerve section. This result suggests that the suppression of flexor activity had not been a result of permanent rewiring

In summary after the cross-reinnervation of antagonistic forelimb muscles, in 15 of 19 animals there were two bursts of EMG activity per step cycle. The periods of activity were those normally associated with the foreign flexor nerve and the original extensor nerve. Indeed the MhT's of most animals were found to be reinnervated by both nerves. However the MhT's of the 4 remaining animals exhibited only an extensor burst during locomotion, although their muscles were also found to have been reinnervated by both nerves.

This small group of animals is, nonetheless of considerable interest, as it indicates that changes in motor output can occur even in such a basic behavior as locomotion. These

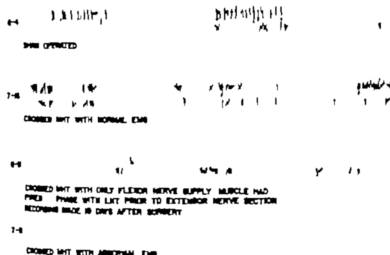


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Acta physiol scand 1978 103, 334–336

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Influence of dehydration on the phasic gustatory response to H_2O, D_2O, and NaCl in the frog (*Rana temporaria*)

By

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Nearly 30 years ago Zotterman (1949) discovered that water applied to the frogs tongue produces a massive volley of large fibre impulses in the glossopharyngeal nerve. His subsequent comparative studies demonstrated the presence of water taste also in several mammalian species (*cf* Zotterman 1956) Taste cells responding rather specifically to water have later been found also in certain insects (*cf* Dethier 1977) This wide-spread species distribution suggests that water taste might fulfil some purpose in the homeostatic control of fluid balance Therefore it was found of interest to study whether dehydration induced by exposure to a salty environment might change the gustatory response to water and NaCl in the frog Slow infusion of deuterium (D O) into the cerebrospinal fluid of goats has been found to inhibit the activity of apparently Na -sensitive cerebral receptors involved in the control of fluid balance (Leksell, Lishajko and Rundgren 1976) For that reason the gustatory response to D_2O was also studied here

Adult summer frogs, *Rana temporaria*, (n = 22) were used for recording of the afferent impulse activity in the glossopharyngeal nerve. Prior to the expts. 12 of the frogs were placed in 1% NaCl solution for about 16 h which caused 5 to 7% reduction of body weight The other 10 frogs were maintained in tap water until they were used for expts. The surgical preparation and the recording technique were principally as described by Zotterman (1949). During the expts. an impulse counter (SE Time-counter SM ?00 Mk2) was continuously registering the nerve impulse frequency every 4th s (duration of each registration 1 s). The 20°C test solutions and the Ringer solution were applied to the tongue for 20 s at a constant flow of 5 ml/min. If the application of fluid happened to be started or discontinued during one of the 1 s registrations, that value was not included when calculating the induced mean increase in frequency Prior to each test the response to the 20 s application of Ringer was recorded, and the observed increase in impulse frequency was subtracted from that obtained during the subsequent test period Hence, the effect of mechanical stimulation of the tongue has been eliminated in the results presented After each test period the tongue was again rinsed with Ringer The test solutions used were Distilled H_2O D_2O (99.8% "Sigma"), 1% (171 mM) and 2% (342 mM) NaCl in either H O or D O The composition of the Ringer solutions was 115 mM NaCl 2.5 mM KCl 1 8 mM $CaCl_2$, 2.15 mM Na_2HPO_4 ($2H_2O$), and 0 85 mM $NaH_2PO_4(H_2O)$ The results obtained were subjected to analysis of variance using BMD Comput. Progr Univ Calif Berkeley 1973

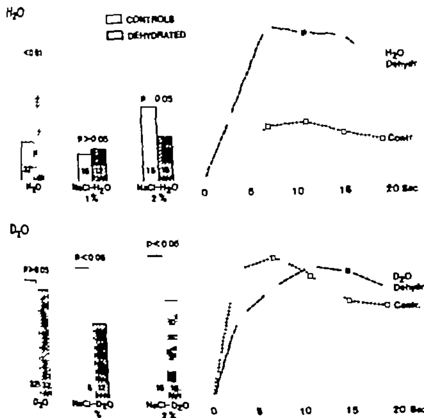


Fig. 1 *Left* Mean increase in glossopharyngeal impulse frequency in control (n = 10) and dehydrated (n = 12) frogs during the 20 s application to the tongue of H_2O, D_2O and of NaCl dissolved in either H_2O or D_2O. To eliminate the influence of mechanical stimulation the mean impulse frequency elicited by preceding 20 s applications of Ringer solution has been subtracted. Figures in the columns indicate numbers of tests. *Right above*: Differences in time course and magnitude for the gustatory response to H_2O in dehydrated and control frogs. *Right below*: Lack of such differences for the response to D_2O.

As shown in Fig. 1 (*left above*) dehydration induced by exposure to a salty environment caused a conspicuous (200 %) increase in the response to H_2O and reduced the effect of 2% NaCl/H_2O by about 40 %. No difference was obtained in the response to 1% NaCl/H_2O between the dehydrated and control frogs. In the control frogs pure D_2O had a much stronger effect than H_2O (Fig. 1 *left below*). However this effect did not become accentuated after dehydration. Also the response to NaCl/D_2O was considerably stronger than that obtained by NaCl dissolved in H_2O. The effects of both 1 and 2% NaCl/D_2O were significantly greater in the control animals than in the dehydrated frogs.

The time courses of the responses to H_2O and D_2O in dehydrated and control animals are shown to the right in Fig. 1. In the control animals the reaction to D_2O was much faster than to H_2O. This was not the case in the dehydrated frogs, however.

Discussion. The fact that the gustatory response to H_2O became considerably accentuated after dehydration lends some support to the idea that the water taste may play a role in the

control of fluid balance of the frog. The manner in which the present frogs were dehydrat raises the question whether diminished amount of body water *per se* or elevated extr and/or intracellular [Na] might have been the crucial cause of increased responsiveness H_2O No determinations of plasma or cellular [Na] were made. However the hyperrea tivity to H O was accompanied by a reduced gustatory response to 2% NaCl (Fig. 1 *k above*) It indicates that elevated bodily [Na] might have been the ultimate cause of th changed gustatory reaction in the dehydrated frogs.

The recorded effects of D O and NaCl/D O were quite different from those obtained l H O and NaCl dissolved in H O The present expts. do not allow any conclusions regardi the influence of D_2O on the taste receptors. However the effect of D_2O appears to be rath nonspecific and not related to the "classical" water taste (Zotterman 1949). This is furth indicated by the fact that no difference in the response to pure D_2O was observed betw dehydrated and control frogs

This wo k was supported by the Swedish Medical Research Council (project 04X-00503).

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Acta physiol. scand. 1978. 103. 337–339

From the Department of Physiology University of Göteborg, Sweden

Note on the conduction velocity of warm afferent fibres from the skin of the human leg

By

Ulf Norrsell and Michael Ullman

Measurements of reaction times to thermal stimuli have been used to investigate the neural substrate of temperature sensitivity in man. The results from these experiments have sometimes generated conflicting theories (*cf.* Sinclair 1967) but the technique remains attractive since it offers the opportunity to correlate subjective experience with objectively quantifiable data. The results of recent reaction time experiments have indicated the subjective experience of "cold" and "warm" to depend on different populations of afferent neurones, and Fruhstorfer (1976) has suggested a conduction velocity in the order of magnitude of 10 m/s for the "cold" afferents, and 2 m/s for the "warm" afferents. The present experiment was made in order to extend our knowledge with regard to the latter group.

We have measured the reaction times to radiation pulses which were applied to different places on one leg of 5 men and 1 woman. The subjects were seated in a comfortable reclining chair with covered eyes and were stimulated on places, which were located between the groin and the ankle and had been painted with ink. They had been instructed to press a manual pushbutton as soon as they felt a sensation caused by the radiation pulse which was generated by emitting the radiation from a 250 W Philips soldering lamp intermittently for 0.1 s through a rotary shutter. The stimulation intensity was altered by changing the lamp voltage and the radiation was directed to strike the skin perpendicularly on a circular area of 40 mm diameter with an intensity gradient of 2/1 from the centre to the periphery. The reaction time was measured in milliseconds between the beginning of the radiation pulse and the pressing of the pushbutton.

Fig. 1 shows the reaction times obtained from the stimulation of a place located ventrally on the thigh 15 cm below the groin in one subject plotted against the stimulation intensity. The stimuli were applied at 60 s intervals and the figure shows that the reaction times were quite constant over a wide range of intensities but increased towards the lower end, close to the threshold for the responses. Stimuli of intensities below the threshold were introduced at random in all the testing sessions and served the double purpose of indicating the approximate sensitivity of the stimulated place as well as the specificity of the test, i.e. that reaction times to the radiation pulses were being measured and not those to parallel acoustical cues. The subjects were asked to describe their subjective experience of the stimulus after each trial and the descriptions fell into 3 categories, which have been indicated by different symbols in Fig. 1. The mean value and standard deviation of the reaction times to stimuli of the intensities which elicited purely warm sensations, i.e. those represented by circles in Fig. 1, have been plotted in Fig. 2 D against the estimated distance from the dorsal roots together with the corresponding data from 3 more places on the same leg, which were in-

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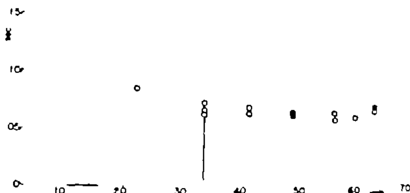


Fig. 1 Reaction times (ordinate) of manual pushbutton responses to 0.15 s radiation pulses which applied to the right thigh of one person, plotted against the amount of energy of the individual ... (abscissa). The different stimulation intensities were used in random order. The stimuli sometimes failed to elicit responses, *vertical bars*, and otherwise elicited 3 different kinds of sensations qualitatively ambiguous *triangles*: warmth, *circles*: warmth plus prickling, *star*. The energy values are based on radiation measurements.

vestigated during the same session (*cf.* also legend Fig. 2). Data which were obtained in the same way from the 5 other experimental subjects are also presented in Fig. 2, and the figure shows that in all instances the reaction times regressed approximately linearly in relation to a diminished distance between the place of stimulation and the central nervous system.

The absolute magnitudes of reaction times to thermal stimuli are correlated to several factors (*cf.* Sinclair 1967), three of which could explain the observed regression: 1 the magnitude of the sensations caused by the radiation pulses, 2, the distance between the skin surface and the warm receptors, 3, the conduction velocity of the primary afferent nerve.

1 The reaction times to stimuli causing greater sensations are shorter than those caused by ... stimuli. In the present experiment, however, the stimulation intensities were altered over the whole range between threshold and pain at each site and thus the shorter reaction times from the proximal sites can only be explained in this context by a greater density of innervation of these sites compared to those located more distally. Sensations of warmth are very dependent of both spatial and temporal summation (Marks, Stevens and Tepper 1976), however, and a differential innervation of the different sites thereby would produce different response thresholds. No such variation between the thresholds of the different stimulation sites was observed.

2. The progress of thermal energy in the human skin has been estimated between 0.5–1 mm/s (Hensel 1952). If the conduction time of the afferent fibres is disregarded temporarily, then the differences between the various reaction times of Fig. 2 could be explained by a delay caused by the warm receptors of the skin of the ankle being located 0.5–1 mm deeper than those of the thigh. Nothing is known about such a variation in depth of the warm receptors, but if existing it ought to be reflected by a corresponding variation of the thickness of the skin. In the human leg the dermis appears to be equally thick in all places whereas the epidermis has been found ... ver ... 70 μm thicker on the shank than on the thigh (Southwood 1955, Whitton and Everall 1973). That difference is obviously too small to explain the variation of the reaction times shown in Fig. 2, which therefore could depend mainly on the delay caused by the conduction time of the afferent nerves.

3 If the sensations of warmth caused by the radiation pulses are transmitted via a homogeneous afferent nerve population and if the two factors which were discussed above can be considered of minor importance, then it should be possible to extrapolate the conduction velocity of these afferent nerves from the present data. It was found to be in average 0.9 m (s 0.2) for the subjects of Fig. 2 when calculated with linear regression (least squares method) on the basis of all the data which were used to construct that figure. This conduction velocity

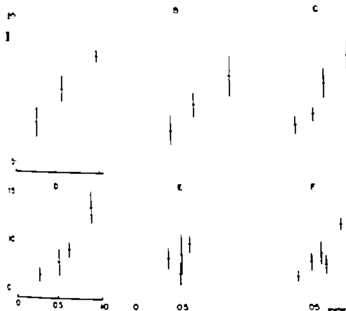


Fig. 2. Mean values, dots, and standard deviations, bars (N 8–21) of the reaction times to radiation pulses applied to 3–6 different places on the right legs of 6 persons. Each graph represents one subject and all data collected during single session. Only the reaction time values from stimulations which resulted in unambiguous sensation of warmth have been used, and the first value of this group from each place of stimulation has been excluded. The different places were stimulated one at time and the first place to be stimulated was always one on the middle of the leg. The distances to the different places of stimulation (abscissa) were measured from right angle projections on the longitudinal axis of the leg, which had been extended to an imagined zero located at the top of the sacral bone.

fits with that of the few specific human warm afferent fibres which have been measured electrophysiologically (cf. Hensel 1976), and the present observations thus are taken to indicate that the sensations of warmth, which are elicited by radiation pulses in the human leg, are transmitted in unmyelinated primary afferents with conduction velocities of that order of magnitude.

This work was supported by the Swedish Medical Research Council (project no. 2857).

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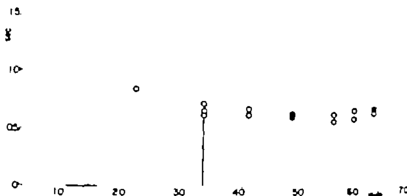


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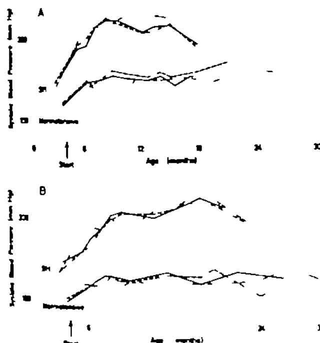


Fig. 1 Mean systolic blood pressure measured indirectly from the tail of spontaneously hypertensive (SH) and normotensive rats as function of age Control —— 85 dB SPL noise —— 105 dB SPL noise: — Measurements are made before the animals were introduced into the boxes (at 4 months of age) and with one month interval during the first year thereafter with 3 months interval. Each value is based on four daily measurements on each rat during 5 days A males, B females.

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Averaged over all measurements taken each group during the first year after the start of the exposure, blood pressure for normotensive males was 150, 155, and 145 mmHg for environments A, B, and C respectively. The corresponding values for normotensive females were 130, 125, and 120 mmHg; for the spontaneously hypertensive males: 205, 210, and 215 mmHg; for spontaneously hypertensive females: 200, 210, and 200 mmHg.

There was no significant difference between any of the groups (one-way analysis of variance p 0.01). Fourthly the spontaneously hypertensive males and females seem to undergo a more rapid initial increase in blood pressure in the 105 dB SPL noise than in the 85 dB or in control environments.

In order to clarify this latter point another 40 spontaneously hypertensive rats (20 in A and 20 in C) were later introduced into the experiment. Fig. 2 shows the average systolic blood pressure over the first 7 months for these two groups. There is no difference between exposed and non-exposed rats, either in males or in females.

The lack of effect of noise on blood pressure in this experiment may seem surprising with regard to the well-established short term effects and the earlier presented effects of intermittent noise over longer periods. Three possible explanations can be proposed. First, the

Acta physiol. scand. 1978. 103. 340-342

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Noise and blood pressure. Effect of lifelong exposure in the rat

By

Erik Borg and Aage R Møller

Is noise a health hazard as far as non-auditory effects are concerned? Two contradict opinions prevail Kryter (1976) is very skeptical toward there being any harmful effect noise outside those on the ear Lehmann (1957) and Jansen (1973), on the other hand, go far as to suggest criteria for noise with respect to deleterious effects mediated through vegetative nervous system (especially those on the cardiovascular system). In the pres work the hypothesesis has been tested that meaningless noise can permanently influen cardiovascular homeostasis. The cardiovascular system has been chosen as a model s it is known that noise exposure of a short duration significantly alters e.g. blood pressu heart rate, and peripheral vascular tone (see e g Hallbäck and Folkow 1974 Borg 197 Furthermore, disturbances in this system are likely to have generalized effects relevant to the concept of health.

In studies over a few months a rise of the blood pressure has been observed in rats dur exposure to intermittent noise (Medoff and Bongiovanni 1945 Buckley and Smookler 1970 In those experiments sudden noise bursts were presented either during the daytime alo (sleep-time for rats) or both day and night. Acoustic stimulus having such a tempor pattern forms an unsatisfactory model for human noise exposure, especially with regard t industrial work In this latter situation noise is more or less continuous during the activ time of the day while sleep-time is relatively noise free.

The aim of the present investigation was to study the influence on blood pressure o lifelong exposure to a varied, meningless noise presented during the greater part of th waking time of the rat (night)

A total of 130 rats of two strains were used in the present study normotensive Sprague-Da ley rats an spontaneously hypertensive Wistar rats (Okamot strai) From n age of 3-4 months they ere kept i one of three so nd-insulated bo es, each with one specific acoustic environment. Systolic blood pressure was determined by a "tail-cuff" technique (Borg and Jansson 1978) and hear g loss as established after one year

Environment A Background noise produced by the rats themselves, approximately 50 dB (A).

Environment B Noise exposure at 85 dB SPL

Environment C. Noise exposure at 105 dB SPL

The noise was presented through 4 Lansing L75 horn in each box It consisted of a 1 640 Hz broad noise band sweeping from 3 to 30 kH t rate of 0.5 H with d ty cycle of 50 %, rise and decay time of inter ruption being 50 ms. The maximum sound energy in the ra ge of 3-15 kHz was 85 and 105 dB SPL. Furthermore, the noise was randomly interrupted by a relay 7 times each night with a total pause time of 2 h.

The mean systolic blood pressure as a function of age is illustrated in Fig. 1 for exposed and non-exposed rats of both strains (A males, B females) 4 main observations can be made. First, the expected difference between normotensive and spontaneously hypertensive

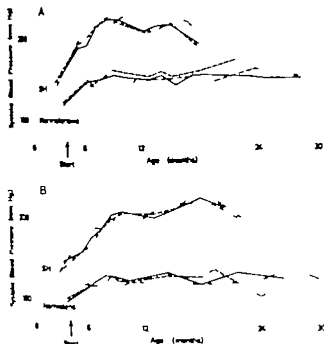


Fig. 1. Mean systolic blood pressure measured indirectly from the tail of spontaneously hypertensive (SH) and normotensive rats as function of age. Control —— 85 dB SPL noise ---- 105 dB SPL noise — Measurements were made before the animals were introduced into the boxes (at 4 months of age) and with one month's interval during the first year, thereafter with 3 months' interval. Each value is based on four daily measurements on each rat during 5 days. A males, B females.

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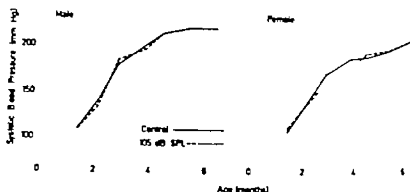


Fig. — Average systolic blood pressure for spontaneously hypertensive rats in control environment (— and 105 dB SPL ( ). Arrow indicates start of exposure.

animals have become deaf. Secondly, the noise was too weak to give an effect, or outside a hypothetic effective range. Thirdly, the rats habituated completely to the n

The auditory thresholds for the rats were measured with a behavioral technique (co tioned suppression) after one year in the noise environment. The animals in environme had a 10–15 dB hearing loss at 6 kHz and those in environment C had a larger loss, 40–60 the value being less for normotensive than for SH rats. In spite of this hearing los SH rats, deafness was not the explanation for lack of long-term effects. The sec possibility is unlikely since the noise, by giving a hearing loss, evidently is harmful to rat. The third possibility, habituation, is the most likely explanation for the present lac effect, and it is supported by a rapid decline of behavioral reaction to the noise.

In conclusion, lifelong exposure, for 10 h daily, to the 85 dB SPL or 105 dB SPL n used in the present study does not alter blood pressure in normotensive or spontaneo hypertensive rats. The present experiments do not exclude the possibility that blood pres may rise in an environment where noise is not neutral, but a warning signal for nox events.

This study was supported by grant No. 74/24 from the Swedish Work Environmental Fund. We thank Agneta Jansson for her skilful technical assistance.

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Acta physiol. scand. 1978. 103. 343–345

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Dopaminergic control of antral gastrin and somatostatin release

By

Kerstin Uvnäs-Wallensten, Jan M. Lundberg and Suad Efendic

In present studies in our laboratory concerning the postulated antral inhibitory influence on the acid gastric secretion we perfused antral pouches of conscious dogs with 0.1 M HCl. We then frequently observed an increase of the peripheral gastrin levels. If anything, we had expected a fall of the level of the circulating gastrin on perfusion of the antrum at this low pH. Since we observed that an increase of the gastrin output often coincided with signs of nausea and vomiting we suspected a nervous mechanism behind the increased gastrin release. It was decided to mimic the state of nausea and vomiting by giving apomorphine. I.v. injections of apomorphine (0.01 and 0.1 mg/kg) caused intense vomiting in the dogs and also an increase of the peripheral gastrin levels. Larger amounts of gastrin appeared to be released by the higher dose of apomorphine (Fig. 1, Table I). Apomorphine is supposed to elicit its effects via stimulation of dopamine receptors. The neuroleptic drug haloperidol is considered as a rather specific dopamine receptor blocking drug. When (n = 4) apomorphine was given 30 min after haloperidol (0.1 mg/kg i.) the gastrin release response induced by the lower dose of apomorphine (0.01 mg/kg) was almost abolished while the response to the higher dose (0.1 mg/kg) was reduced to about 50% (Table I). After haloperidol no vomiting or nausea was observed.

In order to characterize further the gastrin release response induced by apomorphine,

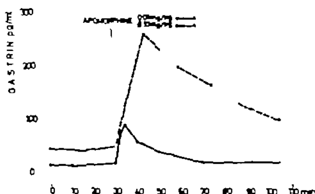


Fig. 1. Release of gastrin following i.v. injection of apomorphine (0.1 and 0.01 mg/kg) in dogs. Note the larger increase of peripheral gastrin levels after injection of the higher dose.

TABLE I Maximal peak increase of peripheral gastrin levels observed in dogs after i.v. injection of [illegible] morphine before and after administration of haloperidol 0.1 mg/kg.

Exp no.	Dose of pomorphine	Gastrin release response pg/ml		
		Before haloperidol	After haloperidol	Per cent of initial response
1	0.1 mg/kg	70	100	37 } 47
2	0.1 mg/kg	180	100	56
3	0.01 mg/kg	105	5	5 } 11
4	0.01 mg/kg	85	15	18

this substance was given in acute experiments to cats. On vagal stimulation in cats and gastrin is known to be released not only into the circulation, but also into the antral lum[illegible] (Uvnäs-Wallensten and Rehfeld 1976 and Uvnäs Wallensten 1977) Since also somatost[illegible] is released into the antral lumen (Uvnäs-Wallensten *et al* 1977), we studied the effect [illegible] apomorphine on both gastrin and somatostatin release. Acute antral pouches (accomplis[illegible] by applying ligatures at the antrum-corpus border and the pylorus) were perfused [illegible] 0.15 M NaCl pH 6 at a rate of 1 ml/min. After having passed the antrum the perfus[illegible] were boiled and analyzed for gastrin and somatostatin like immunoreactivities accor[illegible] to Nilsson (1975) and Elde *et al* (1978) respectively Both gastrin and somatostatin [illegible] released into the antral lumen by apomorphine 0.01–1 mg/kg. 0.1 mg/kg appeared to rel[illegible] the highest amounts of gastrin and somatostatin (Table II). Atropine 0.1 2 mg/kg fa[illegible] to inhibit the apomorphine-induced release of these hormones (n = 2) (Table II).

Thus the present results demonstrate that apomorphine induces an atropine-resis[illegible] release of gastrin and somatostatin from the antrum In the cat electrical vagal s[illegible]

TABLE II Release of gastrin (G) pg and somatostatin (S) pg [illegible] the antral lumen of cats following [illegible] tions of apomorphine 0.001 1 mg/kg. The output of hormones was calculated by multipl[illegible] the gastri[illegible] and somatostat[illegible] levels with the volume of the perfusates (10 ml). To obtai[illegible] output induced by [illegible]pomorphine basal output of gastrin and somatostati[illegible] was subtracted [illegible] total output [illegible]bserved [illegible]fter administration of [illegible]pomorphine

Exp no		Apomorphine mg/kg					
		0.001	0.01	0.1	1	0.01	0.1
1	G	0	16 000	60 000	20 000		
	S	0	15 000	60 000	20 000		
2	G	0	1 000	8 000	5 000		
	S	0	2 000	4 000	0		Atropine 0.2 mg/kg
3	G		30 000			35 000	
	S		5 000			6 000	Atropine 2 mg/kg
4	G		60 000			65 000	
	S		6 000			6 000	

[illegible] release gastrin via an atropine-resistant mechanism (Uvnäs-Wallensten and [illegible] 1977). Nor can the intraantral vagal release of somatostatin be blocked with [illegible] (to be published). Apomorphine is known to stimulate central dopaminergic [illegible], haloperidol to block them (Andén 1967). To explain these observations it might [illegible] be hypothesized that the increased release of these polypeptides induced by apo[illegible] is caused by activation of central dopaminergic neurons.

It has been shown that apomorphine also stimulates the release of growth hormone (La [illegible] et al. 1977). Furthermore, Enochs and Johnson (1976) reported that hypophysectomy [illegible] gastrin release in rats and that the gastrin levels could be restored by injections of growth hormone. At present it cannot be decided whether the release responses observed are secondary to previously released growth hormone, or whether they are mediated via the vagal nerves. As a third possibility apomorphine may activate peripheral dopaminergic receptors. Studies are in progress to investigate the role of the vagus in the apomorphine induced release of gastrin and somatostatin. Preliminary results indicate that the apomorphine induced release of gastrin and somatostatin is virtually abolished following vagotomy.

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Acta physiol. scand. 1978 103 346–348
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"Insulinergic" nerves to the skeletal muscles of the cat?

By

Börje Uvnäs and Kerstin Uvnäs-Wallensten

We have recently presented experimental evidence to indicate the distribution of gastrinergic nerves to the skeletal, smooth and possibly heart musculature of the cat (Uvnäs-Wallensten and Uvnäs 1978) The existence of such extra gastrointestinal sources of gastrin might explain the surprising observation, that in the dog gastrin remains circulating in the blood even after total extirpation of the antrum-duodenum area (Sjödin and Nilsson 1975), which is supposed to contain the major part (>90 %) of the body gastrin. Likewise immunoreactive insulin persists in the blood of eviscerated rats (animals without gastrointestinal tract and pancreas but with intact liver and kidneys) as shown by Smith *et al* (1978) This similarity in the behaviour of the circulating gastrin and insulin after removal of their main glandular sources, prompted us to look for possible insulinergic nerves as extrapancreatic sources of insulin

Cats were killed by an i v injection of nembutal (0 1 g/kg) Perfusates were collected from extirpated hind and fore legs from cats perfused with Tyrode s solution via the femoral and brachial artery respectively For further details of the technique see a concomitant paper in this journal (Uvnäs-Wallensten and Uvnäs 1978). Radioimmunoassay of insulin was performed according to Nilsson (1974).

On electrical stimulation of the sciatic or brachial nerves, insulin-like immunoreactivity appeared in the effluents from the perfused legs, the amounts of immunoreactive material varying with the parameters of the stimuli applied Table I Fig. 1 illustrates the characteristic peakshaped release responses to nerve stimulation. In a concomitant paper (Uvnäs-Wallensten and Uvnäs 1978) we argued for the existence of gastrinergic nerve fibres within the sciatic and brachial nerves. Fig. 2 illustrates the fact that the release of "gastrin" and "insulin" induced by nerve stimulation do not always run in parallel The perfusates from two hind legs of a cat were concomitantly scanned for gastrin and insulin immunoreactivity In the one leg (upper curve) stimuli of high voltage (40 V) and long duration (2 ms) caused a marked release of "insulin" with no "gastrin" release response. The lower curve illustrates the opposite result on stimulation of the sciatic nerve to the other hind leg Stimuli of lower voltage (5 V) and short duration (0.2 ms) induced a marked "gastrin" but no "insulin" outflow Probably the output of "insulin" and "gastrin" are the results of stimulation of different nerve fibres. As was the case with "gastrin" (Uvnäs-Wallensten and Uvnäs *l.c.*) insulin-like material appeared also in perfusates from skinned hind limbs with the circulation of their paws excluded, demonstrating its muscular origin. Prompted by this observa-

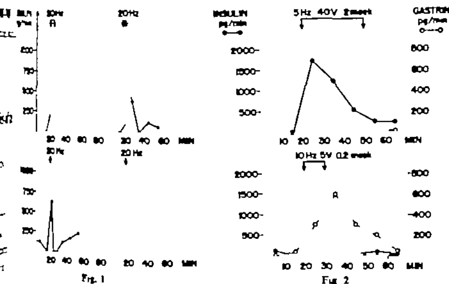


Fig. 1

Fig. 2

Fig. 1 "Insulin" release following stimulation of the sciatic nerve (upper row) and brachial nerves (lower row) at 15 V 2 ms and 20 Hz. The duration of the stimulations were 5, 3, 1 and 0.5 min. Note the larger release following stimulations of longer duration.

Fig. 2 "Insulin" and gastrin release following stimulation of the sciatic nerves in two legs. In this experiment stimulation with 5 Hz 40 V and 2 ms induced a substantial release of "insulin", whereas gastrin was released following stimulation with 10 Hz 5 V and 0.2 ms.

then we looked for releasable insulin-like material also in the heart muscle. In 2 expts. on the perfused cat heart (Langendorff preparation) insulin-like material appeared on electrical stimulation of the vago-sympathetic trunks.

The chemical identity of the insulin-like material released on nerve stimulation has to await the results of studies in progress. Likewise, the structures to which the immunoreactive material is localized have to be identified. There are receptors in the skeletal muscles capable of binding insulin. Theoretically therefore the "insulin" released on nerve stimulation might emanate from such receptors—or some other so far unknown non-nervous storage structures. In our paws perfused with Tyrode's solution no visible contractions occurred as the

Table I. Release of "insulin" from perfused extirpated cat legs on stimulation of the sciatic and brachial nerves.

Exp no	Hz	V	ms	Duration min	Basal output pg/min	Total release during stimulation (pg)
1	20	15	2	5	0	2 500
2	20	15	2	3	0	4 500
3	20	15	2	1	50	1 400
4	20	15	2	0.5	0	0
5	2	15	2	1	25	6 250
6	2	5	2	5	0	8 750
7	5	40	2	30	0	35 000
8	20	20	2	5	0	15 500

result of nerve stimulation The release of the insulin-like material therefore does not s to be secondary to the contraction process. To us one possible source of the released insul might be "insulinergic" nerves. Even if the acceptance of the existence of such nerves requ biochemical and immunochemical identification of insulin in the nerves supplying muscles, some speculations on the functional significance of possible insulinergic" ner might be justified We have so far only experimental evidence suggesting such fibres to skeletal muscles and possibly the heart The purpose of an "insulinergic innervation m be to adapt, via locally released insulin the energy supply of the muscles to their deg of contractile activity Such fibres might supply also smooth muscles, glands etc. A mo precise definition of the functional role of the "nervous insulin" as well as the mechanis of activation of the insulinergic" nerves have to await further detailed studies.

The possible clinical significance of "insulinergic" nerves for our understanding of t nature of diabetes and other metabolic diseases remains to be clarified

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Acta physiol. scand. 1978. 103. 349–351

From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Release of gastrin on stimulation of the sciatic and brachial nerves of the cat

By

Kerstin Uvnäs-Wallensten and Börje Uvnäs

The antrum and the upper part of the duodenum harbour the overwhelming part of the gastrin in the gastrointestinal tract of cat, dog, hog and human (Emås and Fyrö 1968, Nilsson et al. 1973). Electrical stimulation of the cat's vagi causes release of gastrin (identified as heptadecapeptide) concomitantly into the portal blood and the antral lumen (Uvnäs-Wallensten 1977). The vagal fibres responsible for the gastrin release seem to be non-cholinergic since the release response was uninfluenced by atropine (Uvnäs-Wallensten and Andersson 1977). Our assumption that the vagi might contain gastrinergic fibres was supported by the finding of gastrin heptadecapeptide in extracts from the vagi of cats, dogs and humans (Uvnäs-Wallensten et al. 1977). Attempts to visualize vagal gastrinergic fibres by immunofluorescence techniques revealed such fibres in the muscular ages of the stomach (Lundberg and Hökfelt personal communication). Gastrinergic nerves have also been demonstrated in the colon (Uvnäs-Wallensten et al. 1977). Apparently gastrointestinal smooth muscles have a gastrinergic nerve supply. With the idea that a gastrinergic innervation of smooth muscles might reflect a more general biological phenomenon we decided to look for gastrinergic nerves to other types of muscles.

The actual experiments were performed in vitro on hind and fore limbs of cats. The animals were killed with urethane (0.1 g/kg), the extremities extirpated above the knee and the elbow respectively and perfused via the femoral or brachial artery with Tyrode solution at room temperature at a rate of 1 ml/min. The venous effluent was collected in tubes surrounded by ice. Samples, taken at 2 to 10 min intervals were immediately heated for 1 min to 100°C before being frozen and stored. The sciatic or brachial nerves were stimulated by a Grass stimulator, the duration, frequency and intensity of the impulses being varied as indicated in figures and table. The perfusate samples were assayed for immunoreactive gastrin with radio-immunoassay described by Nilsson (1975).

On nerve stimulation immunoreactive material appeared in the perfusates from both hind and fore limbs (Table 1), the amounts released being dependent on the parameters of the stimuli applied (Fig. 1). The release occurred as a single shortlasting peak (around 5–10 min), in spite of continuous stimulation. To exclude perfusion of the skin, in two cats the hind legs were skinned above the ankle and the circulation in the paws excluded by tight ligatures. Immunoreactive material still appeared in the perfusates (Fig. 2), indicating its muscular origin. In vivo immunoreactive material appeared in blood collected from the femoral vein, or in blood from the external facial vein on stimulation of the sciatic and

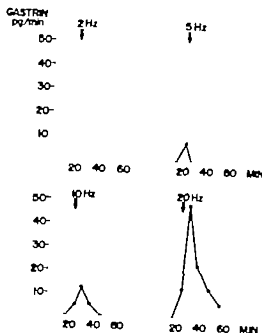


Fig. 1

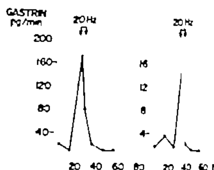


Fig.

Fig. 1 Occurrence of gastrin-like material in perfusates from hind (lower row) and fore limbs (upper row) of cat on stimulation of the sciatic and brachial nerves respectively. Stimulation parameters 15 V ms, ., 5, 10 and 20 Hz. Note increasing release response with increasing frequency

Fig. 2. Occurrence of gastrin-like material in perfusates from two skinned hind limbs of a cat, the paw excluded by metal wire ligatures. Stimulation parameters. 20 V ms, 20 Hz.

the facial nerve respectively (Table I) In the 2 experiments performed, gastrin-like material appeared in the popliteal venous blood on stimulation of the ventral roots L S and S_1.

Since no gastrin cells are known to occur in skeletal muscles we assume the gastrin-like immunoreactive material released on nerve stimulation to originate from gastrinergic nerves although a localization to other gastrin-containing structures cannot be ruled out. Attempts are presently made to visualize the muscle gastrin stores with immunofluorescence technique as also to identify the gastrin variant responsible for the immunoreactivity

The possible occurrence of gastrinergic nerves to both smooth and striated muscle prompted us to look for such nerves also in heart muscle. In two preliminary experiments on the isolated perfused cat heart (a Langendorff preparation) stimulation of the vagosympathetic trunks resulted in the appearance of immunoreactive material (to be published).

The physiological role of a possible gastrinergic innervation of the muscles can at present only be speculated on. It is reported that long term administration of pentagastrin to pregnant dogs and newborn puppies leads to hypertrophic pyloric stenosis in the pups (Dodge 1970) The gastrinergic nerves might therefore influence the contractile machinery or have some "trophic" influence on muscles. Since gastrin administration also has been reported to result in hypertrophy of the parietal cell mucosa of the rat (Johnson 1974) one might imagine gastrin as a hormonal and nervous "trophic" or "stimulating" factor of general biological significance, influencing growth (and) or activity in muscles, glands and other metabolically active tissues and cells.

The antrum-duodenum area is assumed to be the main source of circulating gastrin. This gastrin has a rapid turn-over with half lives of the various gastrin components of <30 min. It has therefore been surprising to find gastrin remaining in the blood after extirpation of the antrum-duodenum in dogs (Sjödin and Nilsson 1975) One reason for the persistence

Table 1. Occurrence of gastrin-like material in perfusates and blood on electrical stimulation of the sciatic nerve, the brachial nerves, the facial nerve, and ventral roots L_7, S_1 and S_2.

Exp.	Stimulation parameters Hz	V	ms	Duration min	Basal release pg/min	Total release during stimulation (pg)
Perfusates from amputated cat limbs						
1	20	20	2	5	10	1 300
2	10	20	2	5	2	50
3	20	20	2	5	2	30
4	2	15	2	1	0	0
5	5	15	2	1	0	40
6	10	15	2	1	0	120
7	20	15	2	1	0	700
8	20	15	2	5	0	1 000
9	20	20	2	5	0	1 800
10	2	5	2	5	0	75
11	10	5	2	5	0	200
12	10	15	0 2	5	0	4 000
Blood from hind leg in vivo (stimulation of sciatic nerve)						
13	10	20	0 1	5	5 (pg/ml)	1 000
Blood from external facial vein (stimulation of facial nerve)						
	10	20	2	5	7 (pg/ml)	150
Blood from hind leg in vivo (stimulation of L_7, S_1, S_2)						
	20	10	0 5	5	20 (pg/ml)	400
	20	10	2	5	30	300

[illegible] of gastrin in the blood after removal of these main gastrin stores might be the release of gastrin from peripheral gastrinergic nerves.

Added in proof. Gastrin in limb perfusates has been identified as gastrin 17 by Gregory and Dockray (personal communication) and visualized in sciatic nerve fibres by Lundberg and Hökfelt (personal communication).

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ADDENDUM to Ca^{++} sensitive microelectrode: intracellular steady state measurement in nerve cell

BY

G R J Christoffersen and Leif Simonsen

Following the publication in Acta physiol scand. *101* 49.–494 of the communicat entitled Ca sensitive microelectrode intracellular steady state measurement in ne cell we have received a comment from dr J D Owen, Dept. of Physiol., Univ of U Medical Center and prof H Mack Brown, same address, regarding our reference to article A Calcium-sensitive microelectrode suitable for intracellular measurement Calcium (II) activity H M Brown, J P Pemberton and J D Owen, (1976) Analyt Chimica Acta *85* 261–276. The authors find that—to pay proper tribute to their cont butions—the reference to their article should have been placed in relation to the followi paragraph in our communication.

'The electrode was prepared as described by Christoffersen and Johansen (1976) exce for two changes The PVC content was 10 instead of 40 and the external tip diamet was 1 μm instead of 20 μm

It was found in our laboratory before the publication of the last mentioned article the reduction of tip size of the previously described electrode containing 40 % PVC (Christo fersen and Johansen 1976) towards 1 μm decreased the slope of calibration plots an increased electrode resistance and response time beyond practical use, and further tha a reduction of PVC content to 10 % counteracted these effects. Before submission fo publication of our manuscript concerned with intracellular use in neurons of *Helix pomati* the effects of PVC content and tip size on slopes of calibration plots were published (Brown Pemberton and Owen 1976). We therefore confirm what may be read from the referenc cited in our communication that the first published description of these effects is found in the article (Brown, Pemberton and Owen 1976)

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Christoffersen, G R. J and E. S. Johansen Microdesign for a calcium-sensitive electrode *Analyt Chim Acta* 1976. *81* 191 195

Acta physiol. scand. 1978. 103, 353–362

From the Department of Clinical Physiology, Klinikum Steglitz, Berlin-West, and the Department of Physiology and Biophysics, Uppsala, Sweden

Effect of intratubular substitution of Na and Cl⁻ ions on the operation of the tubuloglomerular feedback

By

R. MÜLLER-SUUR and H. U. GUTSCHE

Received 10 February 1976

Abstract

MÜLLER-SUUR, R. and H. U. GUTSCHE. *Effect of intratubular substitution of Na^+ and Cl^- ions on the operation of the tubuloglomerular feedback.* Acta physiol. scand. 1978. *103* 353–36[illegible].

Henle' loops of surface nephrons in rat kidneys were perfused orthogradely with various solutions of different compositions, while the stop-flow pressure (SFP) was monitored in the early proximal tubules as measure of the tubuloglomerular feedback response. Modified Ringer solution or iso-osmotic solutions of NaCl, KCl, LiCl, RbCl, choline-Cl, and Na-acetate used to perfuse Henle loop led to significant SFP decrease indicating an intact operation of the tubuloglomerular feedback mechanism. Increased rates with isosmotic solutions of sodium sulphate, potassium sulphate and mannitol did not yield SFP alterations. In order to estimate intratubular sodium and chloride concentration at the macula densa during Ringer, LiCl, choline-Cl and Na-acetate perfusion, early distal tubular fluids were collected at low and high perfusion rates, corresponding to an absent or maximal feedback response. Analysis was performed by microflame photometry and microchloride titration. The results showed that there does not exist a [illegible] threshold for the early distal sodium or chloride concentration at which the feedback starts to operate. We conclude that the intraluminal signal at the macula densa, initiating a tubuloglomerular feedback response, is not necessarily coupled to a single ion species. Either an unspecific ion transfer or some [illegible] events of ionic interaction with the macula densa cells might operate as the initiating step in the feedback loop.

Key words: Rat kidney, single nephron GFR, stopflow pressure, microperfusion, loop of Henle, macula densa, tubulo-glomerular feedback

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The existence of a tubuloglomerular feedback mechanism, first demonstrated by Thurau and Schnermann (1965) has been confirmed by several laboratories (Hierholzer *et al.* 1972, Knacht, Rector and Seldin 1973, Schnermann *et al.* 1970, Schnermann, Persson and Ågerup 1973, Wright and Schnermann 1974). However single steps of this feedback mechanism are still under investigation. Much controversy exists on the specificity of the triggering factor(s). Originally Thurau and Schnermann (1965) postulated the intratubular sodium concentration at the macula densa as the initiating signal. In consecutive publications rather the sodium chloride transfer through the macula densa cells than the intraluminal concentra-

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Table 1 Compiled data (mean ± S.E.) of stop flow pressure changes during perfusion of Henle's loop with different solutions at rates between 0–50 nl/min. Significance of differences were calculated versus SFP during zero perfusion, *0.05 to 0.001 means P < 0.05 to < 0.001 and versus Ringer perfusion (vertical columns), +0.02 to 0.001 means P < 0.02 to < 0.001 n = numbers of nephrons in which the corresponding perfusion rate was tested.

Perfusate	Number of nephrons	SFP at zero loop perfusion [cmH₂O]	ΔSFP t 10 nl/min [cmH₂O]	20 nl/min [cmH₂O]	30 nl/min [cmH₂O]	40 nl/min [cmH₂O]	50 nl/min [cmH₂O]
1 Ringer	18	51.5 ± 2.2	−2.0 ± 1.3 n = 10	−12.3 ± 1.5 13 *0.001	13.7 ± 1.7 n = 11 *0.001	−10.8 ± 1.6 n = 6 *0.01	−11.2 ± 0.9 12 *0.001
2 NaCl	3	60.0 ± 0.9	± 0 n = 2	−6.0 n = 2	−10.3 ± 2.3 n = 3 *0.05	—	−9.5 n = 1
3 LiCl	6	50.3 ± 3.0	0.5 ± 0.7 5	−9.2 ± 1.2 n = 3 *0.001	−15.2 ± 3.1 6 *0.01	−16.5 ± 3.0 4 *0.02	−15.0 ± 3.7 6 *0.02
4 KCl	6	53.9 ± 1.1	± 0 n = 1	−2.0 n = 2	7.4 n = 2	−10.0 2	−8.8 ± 0.9 n = 5 *0.001
5 RbCl	2	43.7	0.5 n = 1	−3.5 2	8.5 2	13 1	−10.5 2
6 Choline Cl	6	48.5 ± 1.9	−2.8 ± 1.4 3	−6.0 n = 2	6.7 ± 1.8 4 *0.05 +0.02	−6.6 ± 2.1 4 *0.05	6.4 ± 1.5 n = 6 *0.02 +0.02
7 [illegible]	6	42.4 ± 2.7	−0.8 ± 0.6 n = 5	−7.6 ± 2.8 4	−10.3 ± 0.3 n = 3 *0.001	−9.5 n = 1	−9.7 ± 0.5 n = 6 *0.001
8 Na₂SO₄	4	64.9 ± 11.2	+0.4 ± 0.9 3	+1.5 n = 2	0.4 ± 0.5 3 0.001	−2.5 + 1.2 n = 4 +0.01	−0.7 ± 0.9 n = 3 +0.001
9 K₂SO₄	3	54.0 ± 2.4	—	–	+1 n = 1	−1.2 n = 1	+0.7 2
10 Mannitol	11	48.5 ± 2.9	−1 2	−1.5 ± 0.7 5 +0.001	−1.6 ± 1.0 5 0.001	+4.5 ± 1.8 n = 3 +0.001	−0.3 ± 0.9 n = 10 0.001

unstained or 0.2% lissamin stained solutions. No apparent influence on the feedback response was observed.

For distal collection expts. rats were prepared as for the SFP expts. The loop of Henle was perfused from an end-proximal site downstream to a paraffin block in mid-proximal tubules to prevent ultrafiltrate admixture. Collections of tubular fluid from early distal segments were performed using pipettes with 6–10 µm tips filled with stained castor oil. The collection site could be identified by the coloured perfusion fluid. Timed samples were taken proximal to a mobile castor oil block from early distal segments. The suction of the collecting pipette was adjusted so that a constant perfusion pressure was measured at the late proximal site. If possible two collections from one nephron were performed at low

tion was defined as the feedback signal (Schnermann *et al* 1970 Wright and Schnermann 1974) In experiments of Israelit Rector and Seldin (1973) and Burke *et al* (1974) the intraluminal Ca^{+} activity seemed by play a crucial role, which, however could not be confirmed by others (Schnermann and Ploth 1975) The most recent hypothesis by Wright and Persson (1974) and Schnermann Ploth and Hermle (1976) is that feedback responses depend critically upon the rate of chloride transport across or into the macula densa cells. In our first experiments (Müller Suur Gutsche and Hegel 1973) on this problem we could not detect a distinct ionic specificity of the feedback mechanism. The present experiments further analyse the ionic requirements for the sensor of this feedback mechanism and try to examine if there is a distinct luminal concentration of sodium or chloride at which the mechanism starts to operate. Our results show that the feedback regulation of stop-flow pressure is not specifically dependent on changes in sodium or chloride concentrations at the macula densa and that substitution of sodium and chloride by some unphysiological ions left the feedback loop intact

Methods

Micropuncture experiments were performed on male Wistar rats (Fa. Winkelmann, Hannover W Germany) weighing 150–250 g, kept on Altromin R (Altromin GmbH, Lage-Lippe, W Germany) diet as described earlier (Müller-Suur *et al.* 1975) anesthesia by i p Inactin® (Promonta, Hamburg), 100 mg/kg B W., i.v infusion of 0.9% NaCl, starting dose 1 ml immediately after the preparation followed by a sustaining rate of 1 7 ml/h. Under these conditions all animals were antidiuretic. Arterial blood pressure was monitored via the left carotid artery catheter using a pressure transducer (Bell and Howell, type 4-327-L233 Barinstoke, U.S.A) The urether was cannulated (polyethylene PE 50 catheter) and timed urine samples were collected under mineral oil. The left kidney was immobilized in a thermoregulated metal chamber (37°C) and covered with prewarmed mineral oil. The experimental protocol for the tubuloglomerular feedback response consisted of measuring the stop-flow pressure of the early proximal tubulues related to the pressure controlled microperfusion of the loop of Henle at rates between 0 and 50 nl/min, as described in detail earlier (Müller-Suur *et al* 1975)

The equipment for micropuncture, *i e* pressure transducer (Kulite, CPL series) and microperfusion system and the set up for tubular blockade with solid paraffin is described elsewhere (Gutsche *et al* 1975 and Hierholtzer *et al* 1974) The following solutions were used for microperfusion of Henle's loop *1* modified Ringer s solution (130 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 10 mM $NaHCO_3$, 7 mM urea, 0 3 g/100 ml indigo-carmine blue, buffered with TRIS to pH 7 4) *2* NaCl (150 mM NaCl 0 3% indigo-carmine blue), *3* KCl (150 mM KCl, 0.3% indigo-carmine blue), *4* LiCl (169 mM LiCl 0.3 % indigo-carmine blue), *5* RbCl (165 mM RbCl, 0.3% indigo-carmine blue), *6* Choline-Cl (150 mM choline-Cl, 0.3% indigo-carmine blue), *7* Na-acetate (150 mM Na-acetate, 0.3% indigo-carmine blue), *8* Na_2SO (91 mM Na_2SO_4, 75 mM mannitol 0.3% indigo-carmine blue), *9* Mannitol (300 mM mannitol, 0.3% indigo-carmine blue) Mean osmolality of all solutions was 310 mosm/kg. In some pilot expts. the influence of staining of the perfusate was tested by using

Table I Compiled data (mean ± S.E.) of stop flow pressure changes during perfusion of Henle's loop with different solutions at rates between 0–50 nl/min. Significance of differences were calculated versus SFP during zero perfusion, *0.05 to 0.001 means $P<0.05$ to <0.001 and versus Ringer perfusion (vertical column), +0.02 to 0.001 means $P<0.02$ to <0.001 — numbers of nephrons at which the corresponding perfusion rate was tested.

Solutions	Number of nephrons	SFP at zero loop perfusion [cm H_2O]	ΔSFP at 10 nl/min [cm H_2O]	20 nl/min [cm H_2O]	30 nl/min [cm H_2O]	40 nl/min [cm H_2O]	50 nl/min [cm H_2O]
1 Ringer	13	31.5±2.2	−2.0±1.3 −10	−12.3±1.5 13 *0.001	−13.7±1.7 −11 *0.001	−10.8±1.6 6 *0.01	−11.2±0.9 12 *0.001
2 NaCl	3	60.0±0.9	±0 2	6.0 2	−10.3±2.3 −3 *0.05	—	9.5 −1
3 LiCl	6	50.3±3.0	0.5±0.7 5	−9.2±1.2 −3 *0.001	15.2±3.1 6 *0.01	−16.5±3.0 4 *0.02	15.0_3.7 −6 *0.02
4 KCl	6	53.9_1.1	±0 −1	−2.0 −2	7.4 2	−10.0 −2	−8.8±0.9 5 *0.001
5 RbCl	2	43.7	0.5 1	−3.5 2	8.5 2	−13 −1	−10.5 2
6 Choline Cl	6	48.5±1.9	2.8±1.4 −3	−6.0 −2	6.7±1.8 −4 *0.05 0.02	−6.6±2.1 −4 *0.05	6.4±1.5 −6 *0.02 +0.02
7 Na-acetate	6	42.4±2.7	0.8±0.6 −5	−7.6±2.8 4	−10.3±0.3 −3 *0.001	9.5 1	−9.7±0.5 −6 *0.001
8 Na_2SO_4	4	64.9±11.2	+0.4±0.9 −3	+1.5 −2	−0.6±0.5 3 +0.001	−2.5±1.2 4 0.01	−0.7±0.9 −3 0.001
9 K_2SO_4	3	54.0±2.4	—	—	+1 1	−1.2 1	+0.7 −2
10 Mannitol	11	48.5±2.9	−1 −2	−1.5±0.7 −5 +0.001	−1.6±1.0 5 0.001	+4.5±1.8 3 +0.001	−0.3±0.9 −10 0.001

unstained or 0.2% lissamin stained solutions. No apparent influence on the feedback response was observed.

For distal collection expts. rats were prepared as for the SFP expts. The loop of Henle was perfused from an end-proximal site downstream to a paraffin block in a mid-proximal tubulus to prevent ultrafiltrate admixture. Collections of tubular fluid from early distal segments were performed using pipettes with 6–10 μm tips filled with stained castor oil. The collection site could be identified by the coloured perfusion fluid. Timed samples were taken proximal to a mobile castor oil block from early distal segments. The suction of the collecting pipette was adjusted so that a constant perfusion pressure was measured at the late proximal site. If possible two collections from one nephron were performed at low

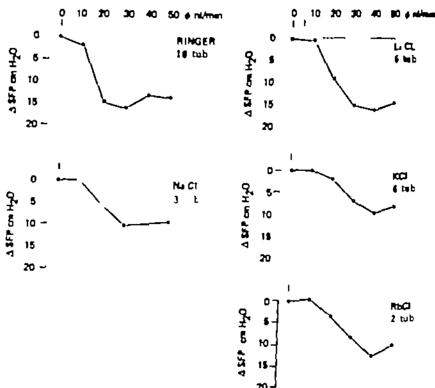


Fig. 1 SFP changes during perfusion of Henles loop with isosmotic Ringers, NaCl, LiCl, KCl, and RbCl. For means ± S.E. see Table I

(10–15 nl/min) and high (50–75 nl/min) perfusion rate. The first collection was randomly done at high or low perfusion rate. Each sample was transferred under water saturated mineral oil and analysed for Na using a microflame photometer (Malnic, Klose and Giebisch 1964) and for chloride by galvanometric titration (Ramsay Brown and Croghan 1955).

In expts. with sodium acetate another approach of the feedback analysis was made. Single nephron GFR (SNGFR) and early proximal flow rate (EPFR) was measured proximal to a solid paraffin block during perfusion of Henle's loop with Na-acetate (at rates of 0 and 60 nl/min) SNGFR and EPFR were calculated from the collected volumes measured in constant bore pipettes and tubular fluid to plasma inulin ratio counting the radioactivity of H-inulin in the tubular fluid sample and artery plasma samples. In these expts. H-inulin was infused at a rate of 75 μCi/h (50 μCi/ml)

The tables compile mean values and ± S.E. significance of differences were calculated with the student's test The SFP at zero perfusion and the ΔSFP at different perfusion rates through Henle's loop were averaged for each nephron from repetitive measurements. Mean observation time in each nephron was 42 min and during this interval the feedback response was elicited 5 to 10 times.

Results

Stop-flow pressure feedback studies

Feedback response by a decreased proximal SFP was obtained at increased flow rate through the loop of Henle with solutions 1–7 whereas perfusion with Na_2SO_4, K_2SO and mannitol

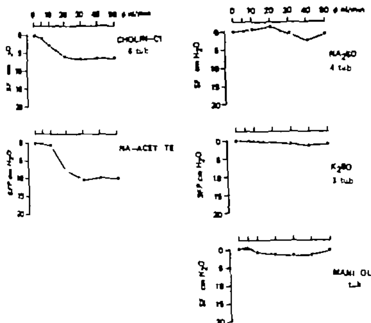
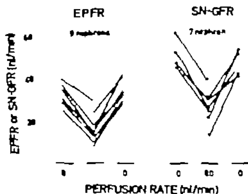


Fig. 2. SFP changes during perfusion of Henle loop with isosmotic choline-Cl, Na-acetate, Na_2SO_4, K_2SO_4 and mannitol. For means ± S.E. see Table I



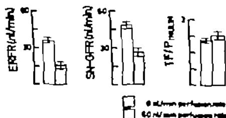


Fig. 3. Early proximal flow rate (EPFR) and single nephron filtration rate (SNGFR) and tubular fluid to plasma ratio of inulin (TF/P) during perfusion of Henle loop with isosmotic ... solution at rates of 0 and 60 nl/min. Above: single experiment data, below mean values ± S.E. ... significantly different (P ...)

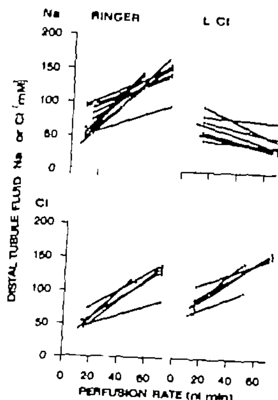


Fig. 4

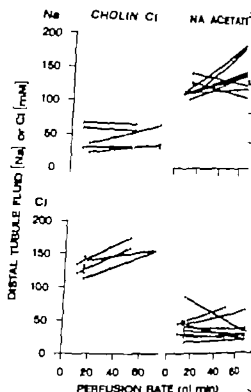


Fig. 5

Fig. 4. N and Cl⁻ concentration i early distal tubular fluid collected at different rates of perfusion through Henle s loop with isosmotic Ringer' and LiCl solution. Lines indicate measurements in the same nephron.

Fig. 5 Na+ and Cl concentratio in arly distal t bular fluid collected at different rates of perfusion through Henle loop with isosmotic choline-Cl and Na-acetate solution. Lines indicate measurements in the same nephron.

had no influence on SFP The data are compiled in Table I The results are depicted in Fig. 1 and 2. Compared with Ringer s the LiCl perfusion (see Fig. 1), yielded slightly but not significantly greater ΔSFP at 50 nl/min loop perfusion whereas choline-Cl at this rate showed a significantly lower ΔSFP compared to Ringer's From these figures it becomes further evident that the onset of the feedback response starts above perfusion rates of 10 nl/min, maximal response was attained between 20 nl/min to 50 nl/min This behavior typical for Ringer s was also apparent in the other perfusion experiments (Fig. 1 and 2)

EPFR and SNGFR measurement during Na-acetate feedback

In 9 nephrons EPFR and SNGFR was measured during Na-acetate induced feedback response or at the state of no feedback stimulus, i e. zero perfusion of the loop Fig. 3 shows the single measurements and the mean ± S E. Under orthograde loop perfusion with Na acetate EPFR and SNGFR decreased to about 50% of the value under zero perfusion. TF/P inulin ratio slightly increased from a mean of 1.3 ± 0 05 to 1.5 ± 0 1 Thus, the observed SFP decreases (Fig. 2) were associated with decreases also of EPFR and SNGFR (Fig. 3)

Table II. Na^+ and Cl^- concentrations in early distal tubular fluid collected during perfusion of Henle loop from [illegible] site downstream solid paraffin block at different perfusion rates (nl/min). *0.02–0.001 gives significance of difference from values at 10–15 nl/min perfusion ($P < 0.02$–0.001) and + gives significance of difference from corresponding values with Ringers perfusion (vertical column) at $P < 0.001$, n = number of nephrons.

Perfusion of loop	mM Na^+		mM Cl^-	
	10–15 nl/min	50–75 nl/min	10–15 nl/min	50–75 nl/min
Ringer n =	73.9 ± 4.9 14	139.0 ± 6.0 14 *0.001	49.3 ± 4.7 8	122.5 ± 5.6 9 *0.001
LiCl n =	71.3 ± 5.6 11	45.6 ± 5.1 9 *0.01	82.8 ± 6.2 6 +	144.5 ± 10.1 5 *0.001
Choline-Cl n =	32.1 ± 5.2 10 +	40.1 ± 5.0 11 +	126.2 ± 5.1 9 +	160.0 ± 3.8 6 *0.001 +
Na-acetate n =	114.5 ± 5.5 10 +	146.8 ± 10.0 10 *0.02	41.8 ± 5.9 10	30.6 ± 3.7 12 +

Distal collection experiments

In a second set of expts. we studied the change of ionic composition of early distal tubular fluid during perfusion of Henle's loop with Ringer, LiCl, choline-Cl and Na-acetate: Increased flow rate of these solutions induced a feedback response (see Fig. 1, 2 and 3). Elevated perfusion rates increased early distal sodium concentration in the case of Ringer and Na-acetate perfusion. The early distal chloride concentration increased in the case of high perfusion rate with Ringer, LiCl and choline-Cl. Single data are given in Fig. 4 and 5 and means ± S.E. are compiled in Table II. It is also apparent from these expts. that an increase of LiCl-perfusion led to a decrease of the distal sodium concentration. During different Na-acetate perfusion rates the distal chloride concentration did not change significantly.

Discussion

The present expts. were designed to define further the chemical composition of the tubular fluid necessary to obtain a feedback response. For this reason loops of Henle were perfused with solutions of different ionic compositions either Na or Cl⁻ free under continuous recording of early proximal stop flow pressure. It could be shown that during perfusion with iso-osmotic NaCl, LiCl, KCl, RbCl, choline-Cl and Na-acetate an intact tubuloglomerular feedback response was observed as found earlier in expts. with Ringer perfusion. Secondly this feedback response could be elicited in the same tubulus during more than 1 hour of repeated stimulation and started only when the perfusion was above 10 nl/min being maximal between 20 and 50 nl/min. Thirdly perfusion with SO containing

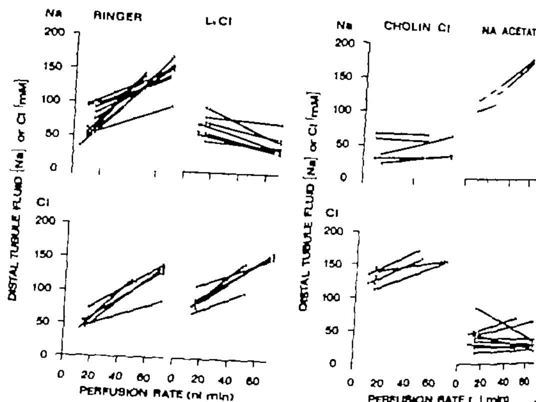


Fig. 4 Na$^+$ and Cl concentratio in early distal t bular fluid collected at different rates of perfusion through Henle's loop with isosmotic Ringer s and LiCl solution. Lines indicate measurements in the same nephron.

Fig. 5 Na$^+$ and Cl$^-$ concentration in early distal tubular fluid collected t different rates of perfusion through Henle's loop with isosmotic choline-Cl and Na-acetate solution. Lines indicate measurements in the same nephron.

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Thus, the feedback response is more likely to be initiated by a passive mechanism at the receptor site. The results of Wright and Persson (1974) that transepithelial hyperpolarization per se induced a feedback response (Wright and Persson, 1974) can also be in agreement with the latter explanation. Morphological support of the hypothesis of passive processes involved into the feedback stimulation can be derived from the studies of Latta, who demonstrated that the morphology of the macula densa cells is different compared to the neighbouring cells of the ascending limb and the early distal tubulus epithelium (Latta 1973). Furthermore, Beeuwkes, Shahood and Rosen (1975) demonstrated the absence of transport enzymes such as Na-K ATPase in macula densa cells. They concluded that the postulated receptor function is not compatible with an active NaCl transport process. Recently in micropuncture studies by Navar and Bell (1977) these authors suggested that the perfusate osmolality may induce the feedback response.

Transmembranal or transepithelial potential difference changes in dependence of the intratubular ionic strength could be one of possible passive steps initiating the tubuloglomerular feedback response. However the present studies cannot discriminate between the above-mentioned concepts. Direct experiments on surface macula densa cells and further histochemical analyses of single macula densa cells are needed to further clarify the mechanism.

Technical assistance is given by L. Lichtenstein, S. Lüdersitz and H. Siebe

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solutions (sodium and potassium sulphate) and mannitol did not lead to a feedback reaction These findings are in agreement with reports in the literature (Schnermann, Persson and Ågerup 1973 Schnermann and Hermle 1975 Wright and Schnermann 1974) with the exception of studies with Na-acetate perfusion. Schnermann, Ploth and Hermle (1976) did not observe a feedback response under orthograde Na-acetate perfusion concluded from EPFR recollection expts. Comparing their results with ours the authors argued that parallel changes of stop flow pressure and EPFR need not occur under all experimental conditions.

However in earlier studies no such discrepancies between changes of SNGFR, EPFR and SFP was found (Hierholtzer *et al* 1974 Müller Suur *et al* 1975 Schnermann *et al* 1970 Wright and Schnermann 1974) Also in the present study of Na-acetate perfusion a high flow rate induced parallel changes of SNGFR, EPFR and SFP From our results it can be shown that Na-acetate releases a tubuloglomerular feedback response. As Schnermann, Ploth and Hermle (1976) used EPFR measurements only it seems possible that by changes of fractional reabsorption i.e. T/FP inulin, actual changes in SNGFR remained undetected.

Distal collection expts. at early distal puncture sites in some of the present perfusion studies were performed to analyse changes of the ionic composition under perfusion through Henle's loop Although an increase of perfusion with Ringer LiCl, choline-Cl and Na-acetate led in all cases to a feedback stimulated SFP-decrease, an elevated distal Na^+-concentration was found only with Ringer's and Na-acetate, whereas no change of distal Na^+-concentration was seen with choline-Cl perfusion and a rather decreased Na^+-concentration with LiCl perfusion (see Fig. 4 and 5 and Table II) Together with the fact that under Na_2SO_4 perfusion no feedback response was observed despite increasing distal Na-concentration (Schnermann *et al* 1970) this ion seems not necessarily be required for the feedback activation

The role of the early distal Cl^- concentration changes within the feedback loop is not similarly conclusive from the present expts. However likewise no distinct Cl^- concentration can be postulated from our expts. at which the mechanism starts to operate. Absent feedback response at low perfusion rates can be associated with high distal Cl^- (choline-Cl and LiCl) or low distal Cl^- (Ringer's, Na-acetate) Likewise states with maximal feedback response can be accompanied by low (Na-acetate) and by high distal Cl^- concentrations (Ringer's, choline-Cl, LiCl) Thus, the chloride concentration per se cannot be the triggering factor of the feedback mechanism

Thus, the nature of the flow dependent signal initiating the tubuloglomerular feedback response is yet not clarified However there exist at least two distinct possibilities Wright and Persson (1974) and Schnermann, Ploth and Hermle (1976) suggested that a supposed transport of Cl^- (Burg *et al.* 1973) or at least of a halide by macula densa cells is mediating the feedback mechanism The present expts. in addition are in agreement with the assumption that even acetate anions are included into this combined anion transport-receptor mechanism. Recent studies of Gutsche *et al* on the transport processes of the ascending limbs of Henle in vivo however showed that Na-acetate leaves the tubulus lumen by passive diffusion and not by active transport as Na-chloride and Na-bromide (unpublished data)

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Acta physiol. scand. 1978. 103. 363–369

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Electrochemical forces for chloride transport in the proximal tubules of the rat kidney

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Received 15 September 1976

Abstract

SOHTELL, M. *Electrochemical forces for chloride transport in the proximal tubules of the rat kidney.* Acta physiol. scand. 1978. *103.* 363–369.

The electrochemical forces for chloride transport in the proximal tubule of the rat kidney were studied using micropuncture techniques. Electrical transmembrane potentials were recorded in randomly punctured tubules with Ling-Gerhard electrodes. Chloride activities in the luminal, cellular and interstitial compartments were measured with ion selective micro-electrodes. Electrical potential measurements between cell to interstitium and lumen to interstitium were -72.1 ± 6 mV and $+0.5 \pm 1.4$ mV (mean $\pm$ S.D.) respectively. The calculated chloride concentrations for lumen, cell and interstitium were 133.0 ± 10.3 mM, 8.5 ± 1.0 mM and 99.1 ± 3.2 mM (mean $\pm$ S.D.) respectively. The net electrochemical forces, qualitatively offer a passive chloride ion pathway through the tubular wall and a chloride equilibrium over the luminal membrane seems to exist.

Key words: Chloride activity, electrical potentials, micro-electrodes, chloride transport, rat proximal tubule, rat

Chloride and bicarbonate are the predominant anions of the glomerular filtrate and are mainly reabsorbed in the proximal tubule of the kidney (Windhager 1968). Walker *et al.* (1941) reported a chloride concentration difference near unity across the proximal tubular wall in the early part, increasing to 1.4 in the middle and late parts. This concentration profile has been confirmed by Gottschalk (1963) and Danielson *et al.* (1970). At various times, proximal tubular reabsorption of chloride has been regarded as a passive or active process, depending on the potential difference measurements obtained by different authors. Frömter and Hegel (1966) first showed that no significant potential difference (PD) existed transtubularly which had earlier been reported to be 20 mV lumen negative (Windhager and Giebisch 1965).

Recently it has become obvious that the proximal tubular epithelia of the mammalian kidney under normal functional state, shows a potential difference profile along its length. Rabbit isolated proximal tubules *in vitro* showed a small negative potential which could be inhibited with ouabain (Burg and Orloff 1970, Kokko and Rector 1971). Using

a similar preparation, Kokko (1973) found a small positive potential unaffected by ouabai which was explained as secondary to chloride diffusion. Barratt *et al.* (1974) and Frömter and Gessner (1974) showed that the PD under free flow conditions in the very early pa of the rat proximal tubule *in vivo* was slightly negative and changed to a small positi potential in the middle and late parts. These findings were recently confirmed by Seely an Chirito (1975)

According to the present knowledge regarding chloride concentration and PD profile in the proximal tubule, reabsorption of this ion is likely to be passive (Giebisch 1974), bu the question remains as to whether the transport route is intra or intercellular The aim o this investigation was to study the electrochemical forces for chloride ion movement. Th chloride activities of the proximal tubular lumen, the cell and the interstitium of the r kidney were measured with chloride selective micro-electrodes of the liquid ion exchang type, used recently by Khuri (1974 a) in renal research The electrical potential difference between the compartments were measured with Ling-Gerhard electrodes. These recording enabled calculation of the chemical transmembrane potential differences for chloride.

Methods

Sprague Dawley rats weighing 200–300 g, were used.

Anesthesia was induced with an intraperitoneal injection of Inactin (Chem. Fabr Promonta, Hamburg West Germany) in a dose of 120 mg/kg b wt. Body temperature was maintained at 37°C with a servo controlled heating table

Animals were tracheostomized The left jugular vein was cannulated and infusion of Ringer solution at rate of ml/h and kg b.wt. started A catheter was inserted to the urinary bladder for drainage, in order t reduce the influence of the vesico-renal reflex. The left kidney was exposed via a flank incision and suspende in a lucite cup. The kidney was embedded and immobilized in a solution of agar in saline, except for small part of the dorsal surface, which was superfused with warmed mineral oil. All the experiments were carried out a ordinary Faraday cage. The proximal tubules were identified in UV light using a Ultropak microscope (E. Leitz, GmbH DC330 Wetzlar West Germany) and punctured randomly

The transtubular potential difference (PD_{trans}) and the potential difference of the interstitium (subcapsular space) versus cell (PD_{i-c}) were recorded with Ling-Gerhard electrodes. The chloride activity of the interstitium (a_{Cl}) and the lumen (a^{l}_{Cl}) were measured with double-barrelled chloride selective electrodes. The intracellular chloride activity (a_{Cl}) was measured with single-barrelled selective electrode with a reference electrode positioned the interstitium. During the potential recordings, the infusion was stopped to prevent electrical interference.

The Ling-Gerhard electrode The electrode (Fig. 1 A) consisted of 1.0 mm Pyrex glass capillary pulled to an outer diameter of about 1.0 µm or 0.1–0.5 µm (see results). The tip was filled with 1 M KCl from a catheter introduced into the rear end. The shank of the electrode was connected to a slightly wider glass capillary filled with 1 M KCl, which had an Ag/AgCl-wire glued to the inside with epoxy resin (UHU Plus, 5 Minuten, UHU-Werk, H. u. M. Fischer 758, Buhl, Baden, West Germany). Electrodes with tip resistances of 5–150 Mohms were used. The asymmetry potential between two measuring electrodes was checked in Ringer solution and in the fluid of the subcapsular space before and after every recording. This potential was not allowed to be more than ± 5 mV and had to be stable otherwise the electrodes were rejected. The maximum deviation from the mean output of the recording device was ± 0.5 mV

The chloride selective electrode The single-barrelled electrode was of the same design as the Ling-Gerhard electrode, but the very tip contained a chloride selective liquid of the ion exchange type (Corning Scientific Instruments, Cat. No. 477315 England). Application of the selective liquid from the rear end into the tip was done through a catheter by applying a pressure more than 10 kg/cm² The length of the selective liquid column was about 0.5 mm. The capillary was then filled with 1 M KCl.

The double-barrelled electrode (Fig. 1 B) was made of two glass capillaries. They were electrically heated kept close to each other twisted 180° and then pulled to a tip with an outer diameter of less than 1 µm. One tip was filled with chloride selective liquid and 1 M KCl as described and the other barrel with 1 M KCl only

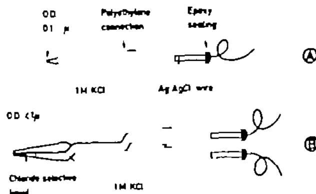


Fig. 1 The electrode design. A) The Ling Gerhard electrode or the single-barrelled chloride selective electrode, when the very tip was filled with selective liquid. B) The double barrelled chloride selective electrode.

Both the single and double electrodes were initially siliconized (Desicote, Beckman Instr. Inc. Fullerton, Cal, USA) to become hydrophobic before the application of the selective liquid. The chloride selective electrodes were kept in air when not used. The electrodes were attached to an electrometer (Vibron Electrometer 33 B, Electronic Instr. Ltd, Richmond, Surrey, England) and a potentiometer recorder (Laboratory Recorder 3053, Yokogawa Electric Ltd, Tokyo, Japan).

The electrodes were calibrated with KCl standards ranging from 5 to 200 mM. The ionic strength of the intracellular fluid is supposed to be the same as that for the luminal and interstitial fluids, and therefore the KCl standards corresponding to the intracellular recordings were corrected with the calculated activity factor of 0.71 (Netter 1959) for an ionic strength of 150 mM. In the following the concentration values were obtained by dividing the activity measured with the factor 0.71. The sensitivity mV/pCl, at 37°C for all the single electrodes ranged from 57 to 60, and for all the double electrodes from 49 to 56. The response time was less than 1 s and the tip resistance around 10^{10} ohms for both electrode systems.

In evaluating the chemical potential differences, Nernst equation was used.

$$E_{Cl} = \frac{RT}{zF} \ln \frac{a_o}{a_i}$$

where E_{Cl} is the diffusion potential, R, T and F have their usual meaning; a_o and a_i express the chloride activity on the respective sides of the membrane.

Results

Measurements were subdivided into 4 series. The first and second series dealt with the PD_{trans} and PD_{i-o}, the third with the chloride concentration of the interstitium $(Cl^-)_i$ and tubular lumen $(Cl^-)_l$ and the fourth with that of the cell $(Cl^-)_c$.

PD_{trans}. The proximal transtubular potential difference was measured with the Ling-Gerhard electrode and the results are shown as a frequency histogram in Fig. 2. The PD_{trans} is 0.5 ± 1.4 mV (mean ± S.D. n = 100) and significantly different from zero ($p < 0.001$).

PD_{i-o}. When the outer diameter of the tip of the Ling-Gerhard electrode was about 1.0 μm, the potential difference of the interstitium versus cell was generally transient and showed a large amplitude scattering. When the diameter was minimized to 0.1–0.5 μm, the potentials became stable and could be recorded for up to 10 min, or until the recording was interrupted for the next puncture (Fig. 3). The distribution of the potential differences observed is presented in Fig. 4. The PD_{i-o} from the second series was -72.1 ± 2.6 mV (mean ± S.D. n = 53).

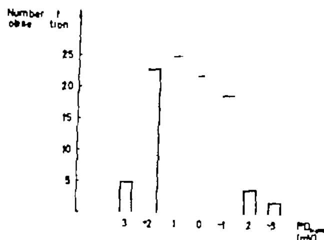


Fig. . The histogram shows the transcellular potential differences (PD_{trans}) measured in the proxi tubule.

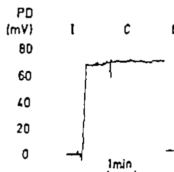


Fig. 3 A single recording of the potential measurements with Ling-Gerhard electrodes. I = interstitium C = tubular cell, L = tubular lumen.

$(Cl^-)_i$ The recordings of the chloride activity in the interstitium were undertaken wit the double barrelled type of electrode. The chloride concentration was 99 1 ± 3.2 mM (mean ± S D n = 9)

$(Cl^-)_l$ The chloride activity of the tubular lumen was also measured using the double barrelled electrode. The recorded value corresponds to a concentration value of 133.0 ± 10. mM (mean ± S D n = 11)

$(Cl^-)_c$ The intracellular chloride activity was measured with a single-barrelled chloride electrode. The potentials recorded were, thus, the sum of the potential for the a_{Cl} and the PD_{i-c} (Fig. 5) The electrodes showed stable sensitivities, mV/pCl while a slight parallel shift of the calibration line could occur Therefore, the a_{Cl} potential was always referred to the actual potential for the $(Cl^-)_i$ of 99 1 mM (see above). The calculated intracellular chloride concentration (Fig. 6) was then 8.5 ± 1 0 mM (mean ± S D n = 60).

The chloride chemical potential differences (E_{Cl}) ± S.E. across the tubular wall, luminal membrane and peritubular membrane were calculated to be 7.9 ± 2.2, 73 5 ± 3.8 and 65 6 ± 3 3 mV respectively By combining the membrane potential PD and E_{Cl} the net electro-

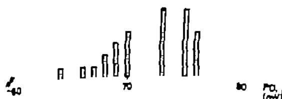


Fig. 4 The histogram shows the potential differences between the interstitium and the tubular cell (PD_{i-c}).

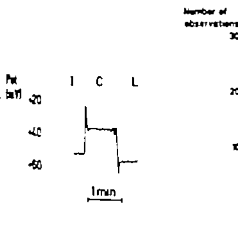


Fig. 5

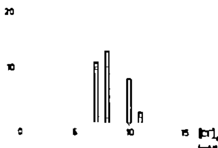


Fig. 6

Fig. 5 A single recording of the chloride activity potential measurement with the single-barrelled chloride selective electrode.

Fig. 6 The histogram shows the calculated intracellular chloride concentration, $(Cl^-)_i$, measured with the triple-barrelled chloride selective electrode.

chemical driving force ($NetE_{Cl}$) ± S.E. over the tubular wall was estimated to be 7.4 ± 2.6 mV which was significantly different from zero ($p < 0.01$). The $NetE_{Cl}$ for the luminal and peritubular membranes were 0.9 ± 4.8 and 6.5 ± 4.2 mV respectively and not significantly different from zero. The estimations of S.E. were done according to Hald (1952).

Discussion

Many technical problems arise in attempting to measure electrical potentials and ion activities in small structures. The difficulties of localizing the electrode tip in the tubular lumen and its orientation, which has been discussed in the literature by Boulpaep (1972) and Frömter (1972), were overcome using a high resolution microscope. In this investigation, when measuring the transtubular PD the electrode tip was placed in the tubular lumen and then withdrawn inside the lumen in the tubular direction about one luminal diameter whereby the recorded potential was constant. Attachment of the tip to the brush-border was easily recognized in the microscope and the potential obtained amounted to about −20 mV which may be responsible for the disparities among the recorded values obtained by different authors.

The results of the PD_{trans} for the rat kidney as recorded by random puncturing of the proximal tubule under free-flow conditions, are in agreement with recently measured transtubular potentials (Barratt *et al.* 1974, Frömter *et al.* 1974, Khuri *et al.* 1974 b, Seely *et al.* 1975). The scattering of the potential values seems to be explained by the potential profile along the proximal tubule (Barratt *et al.* 1974, Frömter *et al.* 1974, Seely

et al 1975) the early part is slightly negative while the middle and late parts become slig positive. The measured values of the PD_{l-c} gave a potential distribution from 0 to 80 r (cell negative) This pattern has been reported previously by Frömter *et al* (1966) a Sohtell and Ulfendahl (1973) The potential scatter seems to be explained partly membrane damages and partly by the likelihood that the electrode tip was located in t cellular membrane. The latter assumption is very plausible in view of the numerous int foldings of the proximal tubular cell. When the size of the tips ranged from 0 1 to 0.5 μ the pattern of the PD_{l-c} recordings altered. When advancing the electrode tip into the c the potential changed abruptly and was in most cases stable for minutes. The mean potent −72.1 mV is in agreement with the values of −74 mV reported by Frömter *et al* (19 and −68.5 mV obtained by Khuri *et al* (1974 b)

The interstitial chloride concentration $(Cl^-)_i$, 99 1 ± 3.2 mM (mean ± S D) was fou to be of the same order in all experiments and the deviation was small. The chlori concentration potential of the interstitium was therefore used as a control of the stabi of the chloride electrode. The random luminal concentration varied between 115 a 150 mM which is in accordance with earlier findings (Walker *et al.* 1941 Gottschalk 196 Danielson *et al* 1970)

The double-barrelled electrode was used initially for a_{Cl} determinations, but stat recordings were not generally obtained This was almost certainly due to leakage betwe the cell membrane and the electrode, as the circumference of the tip was not circular b rather "eight"-shaped For this reason the single-barrelled chloride electrode was used t measure a_{Cl} To date no method exists for measuring different ion concentrations in t cytoplasmic fluid of the proximal tubular cells, a value which is necessary when calculati the ionic strength The calculated activity factor (0 71) is therefore regarded as a reasonab approximated value valid for all renal structures.

Nernst s equation, which was used for the calculation of the electrochemical potential, ca only be used under equilibrium conditions. A maximum potential gradient predicted fc passive diffusion through the cell can then be estimated according to the equation. Seen on the proximal tubule as a unit when randomly punctured the calculated electrochemic forces across the tubular wall which were found significantly different from zero, give passive driving force for chloride movement This movement is also favoured by a hig chloride permeability (Radtke *et al* 1971 Frömter *et al* 1971)

Much discussion has centered around the relative importance of para- and transcellula ion transport in various epithelia *e g* gall bladder (Diamond 1962) In the proximal tubul the electrochemical significance of the intercellular space for chloride transport with its tigh junction near the luminal membrane, has been reviewed by Giebisch *et al* (1973). Thi pathway seems to be responsible for a low tubular electrical resistance, a high ion and water permeability a low fixed charge density and a marked lack of ion restrictivity These properties and the electrochemical driving forces across the tubular wall for chloride ions found in this investigation make it plausible to assume a paracellular pathway for chloride.

The chloride ion transport over the luminal membrane seems to be in an equilibrium state, as Net E_{Cl} was found small and could not be judged to be significantly different from zero as randomly measured in this investigation. This, however does not exclude partial net

chloride movement over the luminal membranes along the proximal tubule according to the in the literature named luminal chloride concentration profile.

This work was supported by grants from the Swedish Medical Research Council and the Medical Faculty of Uppsala, Sweden

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Acta physiol. scand. 1978. 103. 370–378
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Blood velocity in human arteries measured by a bidirectional ultrasonic doppler flowmeter

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Received 16 December 1977

Abstract

RISOE, C. and S Ø WILLE. *Blood velocity in human arteries measured by a bidirection ultrasonic doppler flowmeter* Acta physiol. scand. 1978 *103* 370–378

Blood velocities in 12 arteries were recorded by an ultrasonic doppler flowmeter i 11 young adults Tw major types of velocity patterns existed at rest. In certain arteries (the common carotid, the external caroti the superficial temporal and the proper palmar digital arteries) flow was towards the periphery throughou the entire pulse cycle. Other arteries (the common femoral, the popliteal, the posterior tibial and the ped. artery) exhibited retrograde flow in part of the pulse cycle. In each individual spontaneous variatio between these two velocity patterns was observed in the subclavian, the axillary the brachial and the radi rtery The velocity pattern of each artery is described, and absol te blood velocities at recognizabl instances during the pulse cycle are given. The influence of peripheral resistance on the velocity patter was investigated by reactive hyperaemia of the femoral artery We find that not only is there an upwar displacement of the resting femoral curve relative to the line of zero, but the shape of the velocity patter is lso changed. Our conclusion i that peripheral resistance is of major importance not only for the mea velocity but also for the shape of the velocity pattern i the artery

Instantaneous blood flow patterns have been recorded peroperatively for some time by the use of electromagnetic flowmeters (*e.g.* Schenk *et al* 1960, Gault *et al* 1966, Lauridsen 1968 Dedichen and Kordt 1974). These measurements suffer certain limitations, most notably the unknown influence of anaesthetics, drugs and surgical procedure and the restricted number of vessels available for examination in each patient. The bidirectional ultrasonic doppler flowmeter is used transcutaneously and permits blood velocity recording from a number of arteries without surgery Such transcuteaneously performed measurements do not interfere with physiological conditions. The disadvantages are that the angle between the sound beam and the flow direction has some influence on velocity determination and that volume flow is not yet directly accessible.

Before changes in the velocity patterns with age and under pathological conditions are investigated, a study of young normal adults is needed. Nimura *et al.* (1974) give forward and retrograde peak velocities in 24 heathy individuals, age 22–38 years, for 4 arteries (the

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[illegible] carotid, the brachial the radial and the common femoral), and comment on the [illegible] of two others (the subclavian and the pedal artery). We find their velocities sur[illegible] large and doubt [illegible] whether volume flow if calculated from the mean velocity during [illegible] cycle and an assumed arterial diameter would be within the range of possible flow [illegible]. Our primary aim was, therefore, to repeat these investigations and to establish [illegible] reference velocity values at certain recognizable instances during the pulse cycle [illegible] young normal individuals.

[illegible] *et al.* (1974) describe distinct differences between the velocity patterns of the [illegible], the brachial and the femoral arteries. We have included more arteries in our study [illegible] if all these arteries could be classified in one of Nimura's groups or if other patterns could be observed.

Further Nimura *et al.* (1974) observed that the reverse flow phase was propagated from [illegible] to distal. They suggest that "the reverse flow phase did not result from a rebound [illegible] the periphery" but largely from the nature of the vessel wall, *i.e.* elasticity" To in[illegible] [illegible] contribution peripheral resistance as opposed to elasticity and other qualities [illegible] arterial wall might give to the velocity pattern, we recorded from the common femoral [illegible] at rest and during reactive hyperaemia.

Materials and methods

[illegible] of blood flow velocity were performed on 11 individuals (8 male, 3 female, mean age 24 years [illegible] 23–31), none of [illegible] were known to have any cardiovascular disease. All measurements were [illegible] with the subject in the supine position. 12 arteries were studied at rest, and the common femoral [illegible] pattern [illegible] recorded after [illegible] tourniquet occlusion. An inflatable cuff was applied to the thigh with [illegible] upper edge 10–15 cm below the hip joint. Control registrations were made before inflation. The cuff [illegible] inflated to 200 mmHg for 2 min, [illegible] would give [illegible] 93 [illegible] hyperaemia response compared to 5 min [illegible] (Dedichen and Myhre 1975). All recordings were taken within 15 [illegible] after release of pressure. In [illegible] obtained [illegible] low resistance flow pattern without an increase in heart rate or blood pressure.

The doppler flow meter, [illegible] which can discriminate between forward and retrograde blood velocities ("bi-directional") was built in the department. Parts of the operating technique have been described elsewhere [illegible] 1975). Two sound frequencies are available, 2 MHz and 6 MHz. The emitted beam can be pulsed [illegible]. In this investigation the 6 MHz frequency was used. The bulk of the carotid and the femoral [illegible] was obtained [illegible] pulsed beam, [illegible] recordings from other arteries were achieved by [illegible]. A gel applied to the skin ensured acoustic contact. The probe angle was approx. 45° [illegible] of velocity were made for the apparent angle between the ultrasonic beam and the main direc[illegible] of flow.

Following frequency analysis, the signals appeared on [illegible] storage display unit. An original recording is [illegible] Fig. 1 (photo). All velocities present [illegible] 25 ms sampling interval occurred as [illegible] vertical [illegible]. A long column would reflect the simultaneous presence of [illegible] large range of velocities within the [illegible]. This [illegible] in small arteries [illegible] where the beam would include the entire cross section of the vessel, [illegible] could be observed in larger arteries [illegible] the sampling [illegible] was relatively close to the wall. Doppler [illegible] less than 200 Hz (velocities smaller than 0.035 m/s) would not be detected. By [illegible] switch the [illegible] scale could be doubled or tripled in height. The display was photographed with an instant deve[illegible] film. Maximum linear blood velocity in each sampling was then plotted as [illegible] function of [illegible].

[illegible] have calculated approximate volume flows based on reported mean diameters in x-ray literature and [illegible] estimate of the mean velocity of each artery. The following equations have been used

$$v_{max} = \frac{\Delta f}{2f} \cdot \frac{c}{\cos\theta} \quad (1)$$



Fig 1 An original on-line rec ing from the common carotid tery as it appears on the stor display unit. The display has b photographed with instant d kopi g polaroid film Absc time, ordinate blood veloc. Height of column is proportio to blood velocity The veloc curves presented in this paper present the maximum blood ve city occurring at any instant time.

$$v_{mean} = \frac{v_{max}}{2}$$

$$\bar{v}_{mean} = \frac{1}{T}\int_0^T v_{mean}\,dt$$

$$Q = \pi r^2 \bar{v}_{mean}$$

where Q = volume flow
r = radius
v_{max} = instantaneous maximum elocity across the lumen
v_{mean} = instantaneous mean v locity across the lumen
$\bar{v}_{mean}$ = time average of the instantaneous mean velocity
c = sound velocity
T = duration of the cardiac cycle
Δf = doppler frequency shift
f = emitting frequency
θ = angle between the sound beam nd the flow distribution

By usi g equation (2) w ha e assumed parabolic velocity profile in the artery This assumption is also used by Nimura t al. (1974) nd most other authors, but is questionable, and will be examined in detail in a following paper

Results

Blood flow velocity curves

Two major types of velocity patterns were noted to exist Certain arteries showed "forward" flow (that is flow towards the periphery) throughout the pulse cycle This pattern was observed in the common carotid, the external carotid the superficial temporal and the proper palmar digital arteries

Other arteries exhibited retrograde flow in part of the pulse cycle. A retrograde flow component was always present in registrations of the common femoral, the popliteal, the posterior tibial and the pedal arteries.

In addition there were some arteries which showed a non-steady flow pattern Repetitive registrations of the same individual displayed both of the two patterns mentioned above, though not at the same time. A non-steady flow pattern was demonstrated n the subclavian, the axillary the brachial and the radial arteries.

Time spent in arterial "systole" was 225–300 ms in all registrations except for those of the brachial and radial arteries when retrograde flow was present resulting in a "systolic time of 125–175 ms. When heart frequency was increased by a factor of 1 6 (registrations

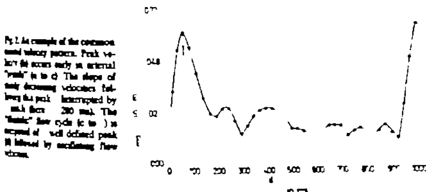


Fig. 2. An example of the common carotid velocity pattern. Peak velocity (b) occurs early in arterial "systole" (a to c). The slope of slowly decreasing velocities following the peak is interrupted by a notch (here [illegible] 200 ms). The "diastolic" flow cycle (c to [illegible]) is composed of a well defined peak (d) followed by oscillating flow velocities.

taken shortly after running), "systolic" time remained unchanged or shortened only by 25 ms.

The common carotid artery. Recordings were done at the level of the cricoid cartilage. The most striking feature of the registrations is that flow is always "forward" that is towards the periphery. Also, flow never falls to zero. The flow cycle can be divided into a "systolic" and a "diastolic" part (see Fig. 2). Peak "systolic" flow velocity (b) was reached in 50-75 ms. During the remaining part of the "systole" flow velocity fell steadily except for a notch half-way from b to c. "Diastolic" flow cycle started with a well-defined peak which was followed by oscillating flow varying both in amplitude and regularity of oscillations. Figures for flow velocity and time relations are presented in Table I.

The external carotid, superficial temporal and proper palmar digital arteries. Measurements of blood velocities in the external carotid artery were taken at the level of the mandibular angle, while those of the superficial temporal artery refer to flow at the level of the external meatus of the ear. The velocity patterns were very similar to that of the common carotid artery. For details we refer to Table I.

Registrations of the proper palmar digital arteries were made close to the distal interphalangeal joint. The flow pattern differed only slightly from the carotid flow pattern. The slope of decreasing maximum velocity following the "systolic" peak was less steep and rarely was there a notch present. The point of end "systole" was less clear-cut, but was followed by a rounded peak as in the common carotid. Peak velocity averaged 0.19 m/s, velocity at end "diastole" measured 0.07 m/s. Time relations matched those reported in Table I.

The common femoral artery. Measurements were performed immediately distal to the inguinal ligament. All registrations showed a forward flow wave with a sharp increase before and a sharp decrease after a high peak (Fig. 3). This was invariably followed by a period of backflow and a second forward flow wave of less magnitude before the flow fell to zero. The part of arterial "diastole" without detectable flow lasted for 150–475 ms. Velocities and time relations are given in Table I.

TABLE I Blood flow velocities and time intervals of the flow cycle in arteries. All figures are based on registrations from 11 individuals, and are median velocity values. Velocity ranges are given in brackets. Where a spontaneous variation in the velocity pattern occurred, the presence of retrograde flow is marked b, while the continuous forward flow pattern is marked f

Artery	Systolic rise time (a to b in ms)	Backflow duration (ms)	Peak systolic velocity (m/s)	End systolic velocity (m/s)	2nd forward peak velocity (m/s)	End diastolic velocity (m/s)
Common carotid	50–75		0.67 (0.40–0.84)	0.13 (0.10–0.26)	0.30 (0.16–0.36)	0.14 (0.08–0.21)
External carotid	50–75		0.48 (0.25–0.65)	0.15 (0.08–0.21)	0.25 (0.11–0.32)	0.12 (0.03–0.17)
Superficial temporal	*50–75*		*0.48* (0.34–0.66)	*0.11* (0.08–0.17)	*0.23* (0.16–0.29)	*0.07* (0.04–0.14)
Common femoral	75–125	125–200	0.38 (0.27–0.47)	0.09 (0.05–0.14)	0.08 (0.06–0.12)	0
Popliteal	*50–125*	*100–250*	0.[illegible] (0.15–0.48)	0.10 (0.04–0.19)	0.06 (0.04–0.15)	*0*
Posterior tibial	50–100	100–200	0.24 (0.19–0.40)	0.07 (0.03–0.13)	0.06 (0.03–0.09)	0
Dorsal artery of the foot	50–100	100–200	0.26 (0.20–0.51)	0.08 (0.05–0.11)	0.05 (0.03–0.16)	0
Subclavian[b]	50–100	50–200	0.65 (0.43–0.78)	0.09 (0.04–0.16)	0.14 (0.06–0.[illegible])	0
Axillary[b]	75–100	50–200	0.31 (0.20–0.45)	0.05 (0.04–0.09)	0.08 (0.05–0.15)	0
Axillary[f]	75		0.33 (0.27–0.45)	0.06 (0.03–0.12)	0.11 (0.10–0.20)	0.05 (0.03–0.09)
Brachial[b]	50–75	75–150	0.38 (0.35–0.76)	0.07 (0.05–0.15)	0.10 (0.06–0.26)	0
Brachial[f]	75		0.54 (0.34–0.73)	0.07 (0.03–0.16)	0.19 (0.11–0.31)	0.06 (0.00–0.12)
Radial[b]	50–75	50–125	0.24 (0.19–0.31)	0.04 (0.03–0.06)	0.05 (0.05–0.07)	0
Radial[f]	50–100		0.31 (0.24–0.52)	0.06 (0.01–0.11)	0.12 (0.08–0.20)	0.05 (0.00–0.10)

The popliteal posterior tibial and pedal arteries The popliteal artery was studied at the knee joint, the posterior tibial at the level of the medial malleolus and the dorsal artery of the foot at a level proximal to the arcuate artery All registrations showed a pattern similar to that of the common femoral artery For details see Table I

The subclavian artery Recordings were made in the supraclavicular triangle. The probe angle was estimated to 60° based on anatomical considerations. All registrations except one showed backflow in part of the pulse cycle.

A subclavian artery registration is presented in Fig. 4 A notch halfway from b to d (as described in the common carotid) often occurred. More than half of the registrations showed a period (25–250 ms) of simultaneous forward and retrograde flow This was observed in other arteries as well but was never as striking as in the subclavian recordings. A second

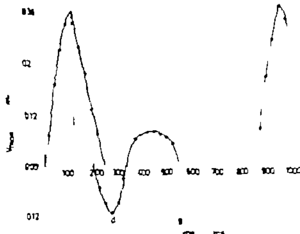


Fig. 3. An example of the common femoral velocity curve. The "systolic" phase (a to d) is composed of a forward flow wave (a to c') through a high peak (b) and the development of retrograde flow (c to d), where the period of simultaneous forward and retrograde velocities (c to c'). A second forward flow wave (e to g) is present, followed by a period with velocities close to zero (g to a). Maximum "diastolic" forward velocity at point f.

wave of backflow occupying 125–200 ms, peak velocity approximately 0.05 m/s, was usually present. For further information see Table I.

The axillary, brachial and radial arteries. Blood velocities of the axillary artery was measured in the axilla. 4 registrations showed an uninterupted forward flow with a carotid type of pattern. The remaining recordings resembled those of the subclavian artery. A second wave of backflow was present in one registration.

The brachial artery recordings were performed at the elbow joint and the radial ones at the wrist. All subjects demonstrated both a flow pattern like that of the femoral artery and a flow pattern resembling the carotid velocity curve. This change in flow pattern occurred without any change in body position or environmental temperature during the period of measurements. When backflow was present, the second forward flow wave was split into two separate peaks in 4 subjects.

Numeric descriptions of all arteries are given in Table I.

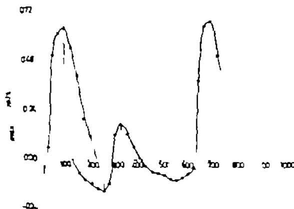


Fig. 4. Plot of a recording from the subclavian artery when backflow was present. The velocity pattern resembles that of the common femoral artery. In more than half the subjects, however, there was a second wave of backflow (g to a) of varying magnitude and duration. The letters used correspond to those in Fig. 3.

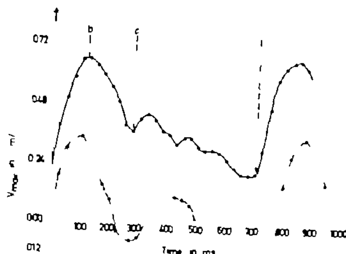


Fig. 5. The velocity patterns o[f the] common femoral artery [at] rest (stipled) and following a t[our]niquet occlusion (drawn) from [one] individual are plotted in the s[ame] diagram for comparison. D[uring] reactive hyperemia all flow [is to]wards the periphery and veloc[ities] are high. The "systolic" time (c) is unchanged [wh]ile the [dia]stole (c to) is slightly shorter. End "systolic" velocity (c) [is] higher than end diastolic contrary to the findi[n]gs at res[t].

Reactive hyperemia (The common femoral artery)

Registrations taken within 15 s after release of a 2 minutes tourniquet occlusion show marked differences from resting femoral flow pattern (Fig. 5) All flow was towards t[he] periphery and the velocity never fell to zero The high peak of the "systolic" forward fl[ow] at rest persisted but compared to resting pattern there was a less sharp decrease in veloc[ity] after the peak was reached. An end "systolic" point indicated a "systolic time of 225-3[...] ms. This was followed by a second smaller peak

Median blood flow velocity was at the "systolic" peak 0 62 m/s, at end "systolic 0.[..] m/s, and at end "diastole" 0.19 m/s. Note that the end "systolic" velocities are higher tha[n] the end "diastolic" values, contrary to the findings of the resting flow pattern.

Discussion

Few reports on instantaneous blood flow patterns are available, and in most cases th[e] registrations are obtained peroperatively by an electromagnetic flowmeter We have foun[d] common femoral velocity curves corresponding closely to the electromagneticly recorde[d] flow pattern reported by Dedichen and Kordt (1974). In the brachial artery we observed a spontaneous variation in velocity pattern at rest as did Gault *et al.* (1966).

Nimura *et al* (1974) have recorded velocity curves in young heathy adults (age 22 3[.] years) by an ultrasonic doppler flowmeter The common carotid, the subclavian, the brachial, the radial, the femoral and the pedal artery were investigated. Generally there is a close correspondence between the shape of their velocity curves and ours. We do not agree, however that blood velocity is low in the pedal artery and all of our recordings of this artery exhibited backflow The most striking difference between the ultrasonic recordings of Nimura *et al* and our registrations is to be found in peak velocity values. Their figures are 2–3 times as high as our findings. We have already pointed out that this will give unreasonably high volume flow values.

Introducing reactive hyperemia by a tourniquet occlusion is a way of selectively lowering the peripheral resistance without increasing the heart rate or the blood pressure. Nor are

TABLE II. Estimated and reference volume flow

Artery	Diameter in cm	Mean velocity in m/s (‾ mean)	Calculated volume flow in ml/min (Q)	Electromagnetic volume flow in ml/min
Common carotid	0.7*	0.21	485	250–450 (1), 500 (2)
External carotid	0.4*	0.19	143	100–200 (1), 150 (2)
Superficial temporal	0.3	0.16	68	
Common femoral	0.85*	0.07	238	228 (3), 239 (4), 320 (5)
Popliteal	0.7*	0.05	116	
Posterior tibial	0.32*	0.04	19	
Pedal	0.25	0.06	18	
Subclavian	0.9*	0.06	229	250 (6)
Axillary	0.7*	0.06[b]/0.07[f]	138[b]/162[f]	160 (7)
Brachial	0.55*	0.06[b]/0.12[f]	86[b]/171[f]	23–107 (8), 7–169 (9)
Radial	0.15	0.04[b]/0.10[f]	23[b]/58[f]	
Proper palmar digital	0.1	0.12	5	

References for arterial diameter marked are taken from Lusca (1974). The figures ithout reference represent our own estimates of vessel diameter.

Figures marked *b* refer to backflow pattern, while those marked *f* refer to continuous forward flow All calculations are based on an estimated angle of 45° between the sound beam and the mean direction of flow, except those of the subclavian artery here approximate angle according to anatomical considerations have been 60°

References for volume flow measured by an electromagnetic flow meter have been: 1 Nordnes, H personal communication, 2. and Krog (1962), 3 Dedichen and Myhre (1975), 4 Vittinen (1975), 5. Lundsen (19 (1972), 7 Dedichen, H., personal communication, 8. Gault *et al.* (1966), 9. Langhorst

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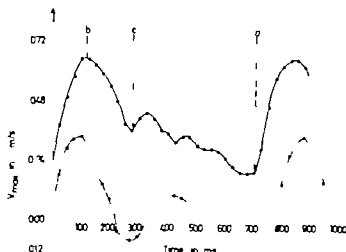


Fig. 5. The velocity patterns of the common femoral artery during rest (stipled) and following a tourniquet occlusion (drawn) from one individual are plotted in the same diagram for comparison. During reactive hyperemia all flow is towards the periphery and velocities are high. The "systolic" time (a to c) is unchanged while the "diastolic" (c to a) is slightly shortened. End "systolic" velocity (c) is higher than end "diastolic" (a) contrary to the findings at rest.

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Artery	Diameter in cm	Mean velocity in m/s (mean)	Calculated volume flow in ml/min (Q)	Electromagnetic volume flow in ml/min
Common carotid	0.7*	0.21	485	290–490 (1), 500 (2)
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Radial	0.35	0.04^b/0.10^f	23^b/58^f	
Proper palmar digital	0.1	0.12	5	

References for arterial diameter marked * are taken from Lucca (1974). The figures without reference represent our own estimates of arterial diameter.

Figures marked b refer to a backflow pattern, while those marked f refer to continuous forward flow. All calculations are based on an estimated angle of 45° between the sound beam and the main direction of flow, except those of the subclavian artery where approximate angle according to anatomical considerations have been 60°.

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properties of the arterial wall changed. If peripheral resistance is of minor importance for the time pattern of the instantaneous velocities, only an upward displacement of the resting femoral curve relative to the line of zero (corresponding to increased mean flow) would be expected in registrations during hyperaemia. However the registrations showed forward flow through the entire pulse cycle, and, most notably end "systolic" velocities are higher than end diastolic" ones. The pattern resembles that of the common carotid. Failure to demonstrate a fully established carotid pattern may be due to a remaining resistance in thigh musculature. Our conclusion is that peripheral resistance is of major importance not only for the mean velocities, but also for the shape of the velocity pattern in the artery.

In some of the arteries where backflow appeared our recordings showed a period (25–150 ms) in which both forward and retrograde blood flow was simultaneously present in the artery. This phenomenon occurred in "systole in diastole or in both, and was also observed by Nimura *et al.* (1974). We believe this is a visible image of the theoretical considerations by Wormersly and McDonald (cited in McDonald 1974). According to their theory the fluid laminae nearest the wall reverse earlier than those closer to the axis of the tube. Peak velocities, however are carried by the laminae midstream.

Estimated volume flows are given in Table II. Our calculations are based on reported mean diameters in x-ray literature and on the calculated mean velocity of all recordings from each artery. A reference to electromagnetic results is given where these are available.

A possible underestimation of the popliteal volume flow must be considered as poplitea blood velocities were recorded with the subjects on their backs and with a knee flexion. Reduced flow in the femoral artery when a hip joint flexion is present has been reporte (Hall 1969).

The authors are grateful to Egil Amundsen and Lars Walloe for working facilities, supervision of the ar and valuable comments on the manuscript. CR was supported by a student fellowship from The Research Council for Science and the Humanities.

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Different effects of the ionophore A 23187 and D-glucose on $^{45}Ca^{2+}$ fluxes in isolated islets of ob/ob-mice

By

JORGE TAMARIT RODRIGUEZ

Received 27 December 1977

Abstract

TAMARIT-RODRIGUEZ, J. *Different effects of the ionophore A-23187 and D-glucose on $^{45}Ca^{2+}$ fluxes in isolated islets of ob/ob-mice.* Acta physiol. scand. 1978. *103*. 379–383.

Fluxes of $^{45}Ca^{2+}$ were studied in β-cell rich islets of non-inbred *ob/ob*-mice, using $LaCl_3$ to wash out extracellular and superficially bound $^{45}Ca^{2+}$. The ionophore A 23187 (10 μM) increased the $^{45}Ca^{2+}$ uptake in islets both at 3 and 20 mM D-glucose, the effect being more pronounced after 10 min than after 120 min of incubation. In incubations for 120 min, 20 mM D-glucose induced higher uptake of $^{45}Ca^{2+}$ than did A-23187. The ionophore enhanced the unidirectional efflux of $^{45}Ca^{2+}$ from preloaded islets. Pretreatment of islets with 20 mM D-glucose in non-radioactive medium inhibited the subsequent D-glucose-induced $^{45}Ca^{2+}$ uptake. Similar pretreatment with A-23187 increased the subsequent ionophore-induced $^{45}Ca^{2+}$ uptake. The results suggest that A 23187 acts by catalyzing Ca^{2+} fluxes across the β-cell plasma membrane. The different effects of D-glucose and A-23187 on $^{45}Ca^{2+}$ fluxes suggest that the two agents act through different mechanisms in the β-cells.

Because stimulation of insulin release is generally assumed to be mediated by Ca^{2+}, several authors have investigated the effects of the Ca^{2+} transporting ionophore A 23187 on insulin release (Ashby and Speake 1975, Charles et al. 1975, Hellman, 1975, Karl et al. 1975, Wollheim et al. 1975, Conway et al. 1976, Somers et al. 1976). The rationale for these experiments has been the assumption that A 23187 affects Ca^{2+} fluxes in the β-cells. However in spite of the fact that data on ionophore effects on insulin release may be of great importance for the interpretation of Ca^{2+} action in secretion, only little is known about ionophore effects on β-cell fluxes of Ca. The aim of the present work was therefore to investigate the effects of A 23187 on $^{45}Ca^{2+}$ uptake and efflux in isolated islets from *ob/ob*-mice. In particular it was decided to compare the effects of ionophore with those of D-glucose, which is thought to induce secretion by affecting Ca^{2+} fluxes.

Materials and methods

A 23187 was a gift from Eli Lilly & Co., Indianapolis, Ind., U.S.A. $^{45}CaCl_2$ was obtained from the Radiochemical Centre, Amersham, Bucks., U.K., and was added in trace amounts to incubation media giving

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specific radioactivity of about 7 8 Ci/mole Dimethylsulfoxide (DMSO) was supplied by Mallinckrodt Chemical Works St. Louis, Mo U.S.A. All other conventional reagents were of analytical grade

Adult non-inbred *ob/ob*-mice of the Umeå colony starved overnight were used for all expts. Pancreatic islets were isolated from the pancreas by free-hand microdissection (Hellerström 1964, Coua ay *et al.* 19 in basal medium at 4°C. The basal medium used in microdissection and subsequent incubations contained in mM D-glucose 3 0 2-amino-2-hydroxymethylpropane-1,3-diol (Tris), 5 0 Na 139 0 K 4.7 Ca 2.6 Mg^{2+} 1.2, Cl^- 151.2 as well as Cl added as HCl to give a final pH of 7 4 A 23187 (10 μM) *added to the medium from a 10^3 times more concentrated solution in DMSO the concentration of DMSO* in the medium was 0 1% (v/v). 0.1% DMSO was routinely included in all control incubation media.

$^{45}Ca^{2+}$ fluxes were studied as previously described (Hellman *et al.* 1976), by measuring the content $^{45}Ca^{2+}$ after washing the islets for 60 min in non radioactive basal medium containing 2 mM $LaCl_3$. Th radioactivity in the islets was expressed in terms of mmol Ca^{2+} with the same specific labelling as in the medium. Samples of labelled medium (5 μl) were used as external standards in the counting procedures

Results

Previous work showed that D-glucose increases the uptake of ^{45}Ca in the La^{3+}-nondisplaceable (intracellular) pool in pancreatic islets from *ob/ob*-mice (Hellman *et al* 1976 In accordance with this finding, Table I shows that islets incubated with $^{45}Ca^{2+}$ for 10 or 120 min in the presence of 20 mM D-glucose contained more ^{45}Ca as compared to islets incubated with 3 mM D-glucose. The addition of 10 μM A 23187 markedly increased the ^{45}Ca + uptake at either 3 or 20 mM D-glucose.

The unidirectional efflux of ^{45}Ca from pancreatic islets was measured by preloading islets with ^{45}Ca + at 3 mM D-glucose for 120 min followed by incubation in non-radioactive media. Fig. 1 shows that 10 μM A 23187 when included in the non-radioactive medium increased the efflux of ^{45}Ca after 10 and 30 min of efflux. The same results were obtained when the ionophore was included during preloading of islets with ^{45}Ca but excluded from the non-radioactive medium (not shown)

TABLE I Effects of A 23187 on $^{45}Ca^{2+}$-uptake Batches of 5 islets were preincubated in basal medium at 3 mM D-glucose at 37°C for 30 min and then incubated for 10 or 120 min i the same medium supplemented with trace amounts of ^{45}Ca (7.8 Ci/mol) and the additives indicated in the table *After incubation with ^{45}Ca islets were transferred to a glucose* and calcium-free basal medium containing 2 mM LaCl and incubated for 1 h t 37°C. Values re mean values ± S E. for the number of experiments given in parenthesis. P < 0.05 P < 0.01 P < 0.005. ‡ P 0.0 when compared to 6.93 ± 0.78. †† P < 0.05 when compared to 9.26 ± 0.96.

Additives	Uptake of labelled calcium (mmol/kg dry wt of islets)	Differences from control
10 min of incubation		
3 mM D-glucose (control)	1 90 ± 0.33 (5)	—
3 mM D-glucose + 10 μM A 23187	5 95 ± 0.78 (5)	4.05 ± 0.74
20 mM D-glucose	2.74 ± 0.36 (5)	0.84 ± 0.23
20 mM D-glucose + 10 μM A 23187	6 01 ± 0.37 (5)	4 11 ± 0.51
120 min of incubation		
3 mM D-glucose (control)	5 06 ± 0.32 (6)	—
3 mM D-glucose + 10 μM A 23187	6.93 ± 0.78 (6)	1.87 ± 0 69
20 mM D-glucose	9.26 ± 0.96 (6) ‡	4.20 ± 0.81
20 mM D-glucose + 10 μM A 23187	11 62 ± 1.26 (6) ††	6.56 ± 1 11

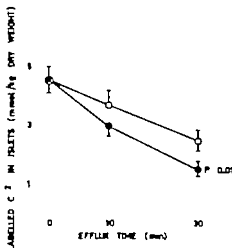


Fig. 1 Effects of A-23187 on efflux of $^{45}Ca^{++}$ from ... islets. Batches of 5 islets were, after pre-incubation in non-radioactive basal medium ... with 3 mM D-glucose, incubated for 120 min in the same medium supplemented ... with trace amounts of $^{45}Ca^{++}$ (0.1 Ci/mol). The islets were then transferred to non-radioactive media ... without ionophore (○) or with 10 μM A 23187 (●) and incubated for the periods indicated ... the figure, followed by incubation with $LaCl_3$ ... Table I. Values are mean values ± S.E. for 5 separate experiments.

D-glucose has been suggested to stimulate the uptake of $^{45}Ca^{++}$ in an intracellular calcium compartment ... with a low turn-over rate and high affinity for the ion (Hellman *et al.* 1976). This idea was further tested in the present study by pretreating islets with 20 mM D-glucose for various periods of time in non-radioactive calcium containing media and subsequently measuring the $^{45}Ca^{++}$ uptake. Table II shows that islets preincubated with 20 mM D-glucose for 10 to 60 min took up significantly less $^{45}Ca^{++}$ in response to 20 mM D-glucose than islets preincubated only at 3 mM D-glucose. Preincubation of islets with 10 μM A 23187 in non-radioactive media for 30 or 60 min did not decrease the subsequent ionophore induced $^{45}Ca^{++}$ uptake but significantly increase it (Table III).

Table II. Effects of preincubation ... with 20 mM D-glucose on the subsequent glucose-stimulated $^{45}Ca^{++}$ uptake by the islets. Batches of 8–9 islets were preincubated at 37°C for 90 min. Controls were preincubated at 3 mM D-glucose during the whole period and tests were treated with 20 mM D-glucose during the last 5, 10, 30 or 60 min of preincubation. The islets were then transferred to medium supplemented with trace amounts of $^{45}Ca^{++}$ (7.8 Ci/mol) and incubated for 10 min followed by incubation ... with $LaCl_3$ as in Table I. Values are mean values ± S.E. for the number of experiments given in parentheses. * P ... 0.02; ** P ... 0.001

Preincubation ... with 20 mM D-glucose (min)	Glucose concentration during incubation with $^{45}Ca^{++}$ (mM)	Uptake of labelled calcium (mmol/kg dry ... of islets)	Differences from control
... (control)	3	1.92 ± 0.10 (9)	−1.09 ± 0.14
	20	3.01 ± 0.13 (9)	—
	20	2.82 ± 0.15 (9)	−0.13 ± 0.16
	20	2.62 ± 0.14 (9)	−0.39 ± 0.12*
... (control)	3	2.06 ± 0.11 (8)	1.12 ± 0.19**
	20	3.18 ± 0.18 (8)	—
	20	2.39 ± 0.10 (0)	0.76 ± 0.12**
	20	2.37 ± 0.15 (8)	−0.80 ± 0.13**

TABLE III Effects of preincubation with A 23187 on subsequent ionophore-stimulated $^{45}Ca^{2+}$ uptake islets. Batches of 5 islets were preincubated in basal medium with 3 mM D-glucose at 3 for 60 min. Controls were preincubated without ionophore and tests were treated with 10 A 23187 during the last 30 min or the whole preincubation period. The islets ere then tr ferred to medium containi g trace amounts of $^{45}Ca^{2+}$ (7.8 Ci/mol) and incubated for 10 followed by incubation with $LaCl_3$ as in Table I Values are mean values ± S E. for the nu of experiments given within parenthesis. P < 0.02. The correlation between time of preinc tion with A 23187 and ^{45}Ca uptake was probably significant (r = 0.553 P < 0.05).

Preincubation time with A 23187 (min)	A 23187 present during incubation with $^{45}Ca^{2+}$	Uptake of labelled calcium (mmol/kg dry wt. of islets)	Differences from control
0	−	1.55 ± 0.15 (6)	− 1.50 ± 0.44
0 (control)	+	3.05 ± 0.57 (6)	—
30	+	3.57 ± 0.22 (6)	0.52 ± 0.63
60	+	5.20 ± 0.80 (6)	2.15 ± 1.33

Discussion

The calcium transporting ionophore, A 23187 has previously been shown to decrease glucose oxidation and ATP content in pancreatic islets (Tamarit Rodriguez *et al.* 19) However the low ionophore concentration employed here (10 μM) does not produce ma metabolic changes in the islets (Tamarit Rodriguez *et al* 1977). The islets used cont more than 90% β-cells (Hellman 1965) and the results are therefore probably representat of this cell type.

Ashby and Speake (1975) reported that A 23187 induced efflux of $^{45}Ca^{2+}$ from prelabell rat islets, a result confirmed here with the β-cell-rich islets of non-inbred *ob/ob*-mice Mo over the present results show that A 23187 stimulates the initial uptake of tracer a enhances the equilibrium content of La nondisplaceable (intracellular) $^{45}Ca^{2+}$ T simplest interpretation of these results is that A 23187 catalyzes fluxes of Ca across t β-cell plasma membranes.

It was previously shown that D-glucose stimulates the uptake of ^{45}Ca + by the La nondisplaceable calcium pool in *ob/ob*-mouse islets (Hellman *et al* 1976) However contrast to the results shown here for A 23187 D-glucose did not affect efflux of ^{45}Ca from prelabelled islets and La^{3+} nondisplaceable Ca taken up in response to D-gluc was not significantly reduced during 90 min of incubation in non-radioactive mediu (Hellman *et al.* 1976). The present results show that pretreatment of islets with 20 m D-glucose led to a decrease of the subsequent ^{45}Ca uptake, suggesting competition b tween $^{40}Ca^{2+}$ and ^{45}Ca in an intracellular calcium pool with low mobility In contra pretreatment of the islets with 10 μM A 23187 rather increased the subsequent ^{45}Ca u take. The clearly different effects of A 23187 and D-glucose on ^{45}Ca fluxes indicate th the two agents act by different mechanisms in the β-cells and thus add some support t the previous notion (Hellman *et al* 1976) that D-glucose-stimulated uptake of La -no displaceable Ca^{2+} may be regulated intracellularly rather than in the islet cell plasma mem branes. This idea does not exclude the possibility that D-glucose also regulates Ca uptak by a La +-displaceable pool located more superficially in the β-cells (Hellman *et al* 1976).

This work was supported by the Swedish Medical Research Council (12x-04756) and J C. Kempe Memori Foundation.

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Acta physiol. scand. 1978, 103, 384–393

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Localization of kallikrein and its relation to other trypsin-like esterases in the rat pancreas. A comparison with the submandibular gland

By

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Received 12 January 1978

Abstract

ØRSTAVIK, T B and G G GLENNER. *Localization of kallikrein and its relation to ot[illegible] trypsin-like esterases in the rat pancreas A comparison with the submandibular gl[illegible]* Acta physiol. scand 1978 *103* 384–393

Kallikrein was located by the direct immunofluorescence technique to the granule-containing [illegible] portion of pancreatic acinar cells. For the demonstration of the intracellular distribution of panc[illegible] kallikrein *in [illegible]o* fixation of the gland was necessary N[illegible] kallikre[illegible] was found in the duct cells or in [illegible] islets of Langerhans. Quantitation by si[illegible]gle radial immunodiffusio[illegible] showed that the concentration [illegible] kallikrein in the pancreas was 132±51 [illegible] g/g wet weight *i.e.* 1/91 that of the rat submandibular gl[illegible] Bz-Arg-OEt-esterases were in the pancreas found as pro-enzyme but as active enzyme in the submandib[illegible] gland Trypsin like esterases, hydrolyzing ε-amino caproic acid napht[illegible] I-AS-D HBr (ACA), were found [illegible] the active f[illegible]rm in both submandibular gland and pancreatic homogenates. The submandibular gl[illegible] contained per g wet weight 6 times as much ACA-esterase activity as the pancreas. In the submandibu[illegible] gland, kallikrein and ACA-esterase activity were f[illegible]nd togethe[illegible] in practically all granular tubular ce[illegible] Thus, the granular tubular cell contains kallikrein as well as other trypsin-like enzymes like the AC[illegible] esterase, and is i[illegible] this way comparable to the pancreatic acinar cell. An e[illegible]traglandular function [illegible] kallikrein is suggested for the pancreas in contrast to other kallikrein-containing exocrine organs.

Glandular kallikreins are found in the kidney and the urine, in the pancreas and the pancre[illegible] tic juice, and in the major salivary glands and the saliva of all mammalian species studi[illegible] (for review see Nustad *et al* 1978). Kallikreins of exocrine fluids probably are specific f[illegible] the organ that secretes them (Nustad *et al* 1975) Rat kallikreins of renal and submandibul[illegible] gland origin have been purified (Nustad and Pierce 1974 Brandtzaeg *et al* 1976), and the [illegible] cellular origin reported (Ørstavik *et al* 1975 Brandtzaeg *et al* 1976, Ørstavik *et al* 197[illegible] Ørstavik 1978). In the kidney kallikrein is present in the distal tubule cells, and in the maj[illegible] salivary glands it is present in the striated duct cells. These two locations have morphologic[illegible] similarities. No kallikrein is found in the acinar cells of the salivary glands, but the granul[illegible]

[illegible] cells of the rat submandibular gland are particularly rich in kallikrein (Ørstavik *et al.* 1977) and in the mouse also other trypsin-like enzymes (Ekfors and Hopsu-Havu 1971). The exocrine portion of the pancreas consists mainly of acinar cells, and the duct [illegible] is poorly developed with no striated ducts or granular tubules. In the pancreas, [illegible]-like enzymes are found in the acinar cells (Hopsu and Glenner 1963), and studies on [illegible] fractions (Bhoola and Dorey 1971) indicate that kallikrein is located within the [illegible] zymogen granules. However the cellular origin of pancreatic kallikrein is unknown. Kallikrein has been purified from the pancreas of several species (for review see [illegible] *et al.* 1978), and the pancreas is the only organ where kallikrein with certainty has been detected as a pro-enzyme (Fiedler and Werle 1967).

The purpose of this investigation was to establish the cellular localization of kallikrein (or pro-kallikrein) within the pancreas. Furthermore, its distribution in relation to other [illegible]-like enzymes in the pancreas as well as the submandibular gland was studied.

Materials and methods

Animals. Female Sprague-Dawley rats, age 12 to 18 weeks, were used. The animals were killed by [illegible] blow [illegible] head. The submandibular glands and the pancreas were extirpated and frozen (−20°C) prior to [illegible]. The animals used in localization studies were anesthetized with nembutal (70 mg per kg [illegible]) and tracheotomized but maintained spontaneous respiration during the experimental period.

Fixation procedure. The pancreas was fixed *in situ* 1) by perfusion through the superior mesenteric [illegible] using [illegible] polyethylene cannula (Portex, PP 25) with manually applied pressure and [illegible] perfusion rate of 1 ml/min, or 2) by retrograde perfusion through the abdominal aorta with [illegible] constant perfusion pressure of 12 [illegible] kPa (130 cm high water column), and [illegible] perfusion rate through the polyethylene cannula (PP 60) of 3 ml/min. When perfusion through the abdominal aorta was performed, pressure was applied [illegible] the [illegible] and was [illegible] to the renal arteries during the perfusion period, and in some animals the renal [illegible] was occluded prior to perfusion. The caval vein was always cut above the kidneys to prevent build-up of back pressure. The gland was first perfused with phosphate buffer saline (PBS, 0.01 M Na-phosphate, pH 7.4, 0.15 M NaCl) containing 5 I.E./ml heparin. Buffer perfusion was maintained until the pancreas was pale (after about 2 min), and perfusion with fixative followed for the next 10 min. The [illegible] of the perfusates [illegible] 22°C. After *in situ* fixation the pancreas was removed and post-fixed in the same fixative for 20 h (4°C). The submandibular gland was extirpated during anesthesia, cut into pieces ([illegible] 4 [illegible] 4 mm³), and fixed *in vitro* for 20 h (4°C). After fixation the tissue was brought through cold [illegible] and embedded in paraffin.

Fixatives. The fixatives employed were 96 [illegible] ethanol, 4% formaldehyde in PBS (pH 7.4), 4% buffered formaldehyde saturated with picric acid, or Helly' fluid (a formaline fixative containing dichromate and [illegible] Pearse 1972). As formalin does not precipitate blood, the wash-out perfusion with buffer was omitted for formaldehyde-perfusion.

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Acta physiol scand. 1978. 103. 384–393

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Abstract

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kallikrein (Brandtzaeg *et al.* 1976), 0.21 mg/ml for the anti-urinary and 0.41 mg/ml for the anti-salivary kallikrein conjugate. The anti-salivary kallikrein conjugate was also absorbed with the cross-reacting esterases A and B from the submandibular gland (Brandtzaeg *et al.* 1976), 0.8 mg/ml. Binding specificity was tested by preincubation of the tissue sections with unlabelled antiserum to submandibular gland or urinary kallikrein followed by incubation with conjugate diluted in the unlabelled antiserum. The antisera were substituted with normal rabbit and sheep sera, respectively for positive controls. On tissue sections of the submandibular gland the anti-submandibular gland kallikrein conjugate was used at an IgG concentration of 0.15 mg/ml. The immunological specificity of the fluorescence reaction was controlled by absorption with 0.41 mg purified submandibular gland kallikrein per ml working dilution of the conjugate.

Histochemistry. Serial sections (6 μm) adjacent to those stained for kallikrein by immunohistochemistry were stained for tryptophan by the Adams DMAB-nitrite method (Pearse 1968) or for trypsin-like esterase activity as described by Hopsu and Glenner (1963).

Quantitation of enzymes in gland homogenates. Paired submandibular glands from 7 animals were homogenized separately. Individual pancreas homogenates were produced from 8 animals. Tissue homogenization and kallikrein quantitation by its antigenic activity in a single radial immunodiffusion (SRID) system or by its Bz-Arg-OEt-esterase activity was performed as described previously (Ørstavik *et al.* 1977). A rabbit antiserum to rat submandibular gland kallikrein was used for the SRID measurements. The antiserum contained 32 precipitating units when tested against a standard submandibular gland homogenate containing 0.19 mg/ml kallikrein. Trypsin-like esterase activity was measured using the ACA-ester substrate as described by Hopsu and Glenner (1963). The purified submandibular gland enzymes kallikrein, esterases A and B (Brandtzaeg *et al.* 1976) as well as purified urinary kallikrein (Nustad and Pierce 19[illegible]) were tested for ACA-esterase activity. Bovine trypsin was used as standard, and ACA-esterase activity was expressed in trypsin units: one trypsin unit (TU) was defined as the amount of free naphtol-AS released per min by 1 μg of the trypsin standard, measured by the optical density at OD[illegible].

Pancreatic pro-enzymes were activated either by auto-digestion (24 h, 22°C) or by trypsin-activation (equal volume of trypsin (1 mg/ml in 0.05 M TRIS/HCl buffer, pH 7.75, 30 min, 22°C) was added to the pancreas homogenate). Trypsin activation was stopped with soybean trypsin inhibitor (SBTI) (10 mg final concentration). The activated homogenate was then left for 30 min (22°C) before testing for esterase activity. Trypsin activation was also done on the submandibular gland homogenates.

Determination of total protein. Total protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Drugs: Heparin (Leo, Ballerup, Denmark), Fast Garnet GBC® (Sigma), Trypsin 1:250® (Difco).

Results

Immunohistochemical localization of kallikrein. In the pancreas, kallikrein was located within the acinar cells (Fig. 1). The kallikrein-specific fluorescence (Fig. 1 c) showed a similar distribution as the DMAB-positive secretory granules (Fig. 1 e) and was found in all acinar cells. No kallikrein was observed in the cytoplasm of the ductal cells, whereas some kallikrein-containing material was seen in the duct lumen and adhering to the luminal wall. The islets of Langerhans were devoid of kallikrein. This location of kallikrein was obtained by use of both the anti-salivary and the anti-urinary kallikrein conjugates. However, the latter was a weaker antiserum and produced faint fluorescence. Staining was completely blocked by absorbing the conjugates with purified submandibular gland or urinary kallikrein (Fig. 1 a and b). Absorption with submandibular gland esterases A and B did not alter the fluorescence reaction. The fluorescence reaction was abolished by preincubation of the tissue sections with the antisera to kallikrein but not with normal sera.

For the immunofluorescence studies on the pancreas, ethanol was the only fixative which gave a strong fluorescence against a dark back-ground. With the other fixatives employed (formaldehyde, formaldehyde with picric acid, and Helly's fluid) there was little difference between specific and non-specific staining and no brilliant fluorescence was seen in the

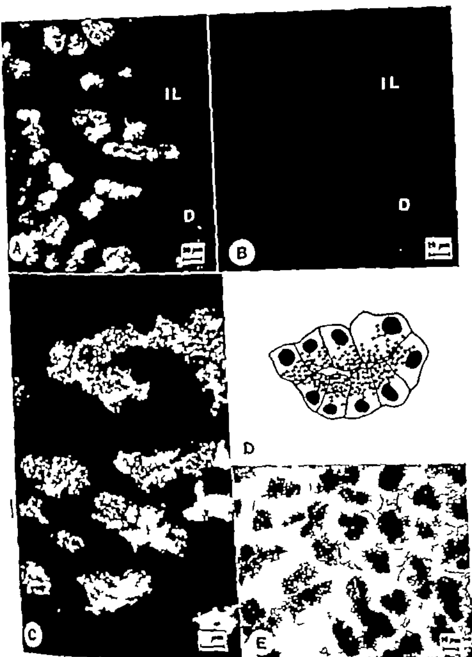


Fig. 1. Immunohistochemical localization of kallikrein in the rat pancreas fixed in situ with ethanol. Kallikrein [illegible] is in the luminal part of all acinar cells (A and C) as visualized by stars in the line-drawing of pancreatic acinus (D). Kallikrein shows [illegible] similar distribution as DMAB-positive granular material in formalin fixed tissue (E). The specificity of the fluorescence reaction is demonstrated in the microphotographs of identical areas of two adjacent sections: one reacted with unabsorbed antisubmandibular gland kallikrein conjugate (A) and the other with conjugate absorbed with purified urinary kallikrein (B). The exposure time for Fig. A and B [illegible] 15 sec. IL—islets of Langerhans; D—duct.

kallikrein (Brandtzaeg *et al* 1976), 0.21 mg/ml for the anti-urinary and 0.41 mg/ml for the anti-sal kallikrein conjugate The anti-salivary kallikrein conjugate was also absorb edwith the cross-react esterases A and B from the submandibular gland (Brandtzaeg *et al* 1976), 0.8 mg/ml. *Binding specific* was tested by preincubation of the tissue sections with unlabelled antiserum to submandibular gland urinary kallikrein followed by incubation with conjugate diluted in the unlabelled antiserum. The antis were substituted with normal rabbit and sheep sera, respectively for positive controls. On these secti of the submand bular gland the anti-submandibular gland kallikrein conjugate was used at an IgG co centration of 0.15 mg/ml. The immunological specificity of the fluorescence reaction was controlled absorption with 0.41 mg purified submandibular gland kallikrein per ml working dilution of the conjuga

Histochemi try Serial sections (6 μm) adjacent to those stained for kallikrein by immunohistochemis were stained for tryptophan by the Adam s DMAB-nitrite method (Pearse 1968) or for trypsin-like esters activity as described by Hopsu and Glenner (1963).

Quantitation f enzymes in gland homogenates. Paired submandibular glands from 7 animals ere h mogenized separately Individual pancreas homogenates were produced from 8 animals. Tissue homogeni tion and kallikrein quantitation by its a tigenic activity in a single radial immunodiffusion (SRID) s) or by its Bz Arg-OEt-esterase activity was performed as described previously (Orstavik *et al.* 1977) T rabbit antiserum to rat submandibular gland kallikrein was used for the SRID measurements. Th antiserum contained 32 precipitating units when tested against a standard submandibular gland homogena containing 0.19 mg/ml kallikrein. Trypsin-like esterase activity was measured using the ACA-ester substrate as described by Hopsu and Glenner (1963). The purified submand bular gland enzymes kallikrei esterases A and B (Brandtzaeg *et al* 1976) as well as purified urinary kallikrein (Nustad and Pierce 197 were tested for ACA-esterase activity Bovine trypsin was used as a standard, and ACA-esterase activ was expressed in trypsin units one trypsin unit (TU) was defined as the amount of free naphtol-AS- released per min by 1 μg of the trypsin standard, measured by the optical density at OD

Pancreatic pro-enzymes were activated either by a to-digestion (24 h, 22°C) or by trypsin-activatio (equal volume of trypsin (1 mg/ml in 0.05 M TRIS/HCl buffer pH 7 75 30 min, 22 C) was added to t pancreas homogenate). Trypsin activation was stopped with soybean trypsin inhibitor (SBTI) (10 mg/ final concentration). The activated homogenate was then left for 30 min (22°C) before testing for estera activity Trypsin act vation was lso done on the submandibular gland homogenates.

Determination f total protein. Total protein was measured by the method of Lowry *et al.* (1951) wi bovine serum lbumin as a standard

Drugs. H parin (Leo, Ballerup, Denmark), F st Garnet GBC® (Sigma), Trypsin 1 250® (Difco).

Results

Immunohistochemical localization of kallikrein. In the pancreas, kallikrein was locate within the acinar cells (Fig 1). The kallikrein specific fluorescence (Fig. 1 c) showed similar distribution as the DMAB-positive secretory granules (Fig. 1 e) and was found in all acinar cells. No kallikrein was observed in the cytoplasm of the ductal cells, wherea some kallikrein-containing material was seen in the duct lumen and adhering to the lumina wall The islets of Langerhans were devoid of kallikrein. This location of kallikrein wa obtained by use of both the anti-salivary and the anti-urinary kallikrein conjugates. How ever the latter was a weaker antiserum and produced faint fluorescence Staining wa completely blocked by absorbing the conjugates with purified submandibular gland o urinary kallikrein (Fig. 1 a and b) Absorption with submandibular gland esterases A and B did not alter the fluorescence reaction. The fluorescence reaction was abolished by pre- incubation of the tissue sections with the antisera to kallikrein but not with normal sera

For the immunofluorescence studies on the pancreas, ethanol was the only fixative which gave a strong fluorescence against a dark back-ground. With the other fixatives employed (formaldehyde, formaldehyde with picric acid, and Helly s fluid) there was little difference between specific and non-specific staining and no brilliant fluorescence was seen in the

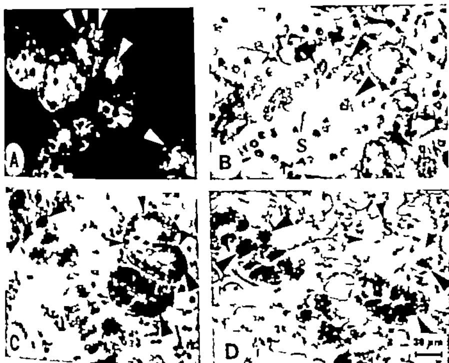


Fig. 2. Distribution of ACA-esterase (B and D) of the rat submandibular gland in relation to kallikrein (A) and tryptophan (C). Small arrows point to non-granular duct cells whereas large arrows point at granular tubular cells. Note the presence of kallikrein and ACA-esterase in the same granular tubular cells (A and B), and the similar distribution of esterase and tryptophan (C and D). S—striated duct.

Total pancreatic and submandibular gland ACA-esterase activity per g wet weight is given in Table I. Pancreatic ACA-esterase activity was not significantly altered by gland homogenate activation (Table I). Similar to the submandibular gland Bz-Arg-OEt-esterase activity (Ørstavik and Gautvik 1977) the difference between the ACA-activity of paired glands was small compared to that between different animals, *i.e.* 5.5 ± 7.1% versus 33.3 ± [illegible]%. The contribution of various submandibular gland esterases to total gland Bz-Arg-OEt-esterase activity and their specific Bz-Arg-OEt-esterase activities are known (Brandtzaeg *et al.* 1976), as well as their specific ACA-activity (Table II). It was thus possible to give an estimation of the percentage of total submandibular gland ACA-activity contributed by other esterases than kallikrein (Table II).

Discussion

The direct immunofluorescence technique has previously been used to localize kallikrein in other exocrine organs (Ørstavik *et al.* 1975, Brandtzaeg *et al.* 1976, Ørstavik *et al.* 1976, Ørstavik 1978). The reliability of these results was substantiated by the corresponding change in kallikrein fluorescence and kallikrein enzyme activity in the developing rat submandibular

luminal part of the acinar cells The *in situ* fixation was absolutely necessary to demon[illegible] the intracellular distribution of pancreatic kallikrein. Also, fixation had to be achie[illegible] rapidly When fixation was not satisfactory the fluorescence observed was weaker [illegible] more uniformly spread out over the whole acinar cell. A satisfactory fixation through[illegible] out the entire gland depended on the wash-out effect of the buffer perfusion to preve[illegible] precipitation of blood by all fixatives except formaldehyde. When a retrograde per[illegible] through the abdominal aorta was used occlusion of the renal vessels improved fixation.

In the submandibular gland kallikrein showed the same localization as previously (Ørstavik *et al* 1975 Brandtzaeg *et al* 1976), *i.e* in the granular tubular and striated cells. Compared to the other fixatives employed, 4 [illegible] formaldehyde saturated with picri[illegible] acid resulted in a brilliant fluorescence with only little nonspecific back-ground staining and individual kallikrein-containing granular tubular cells could easily be differentiated All cells with a typical granular appearance contained kallikrein, even though the intensit[illegible] varied from cell to cell.

Localization of ACA-esterase and its relation to kallikrein. ACA-esterase activity wa[illegible] demonstrated in the granular tubular cells of the submandibular gland. No reaction wa[illegible] detected in the striated duct cells whereas some traces could occasionally be seen within th[illegible] duct lumen. No esterase activity was detected in the acini. Positive esterase activity wa[illegible] only observed in formaldehyde-fixed tissue, rendering comparison with kallikrein-positiv[illegible] cells difficult since in these sections cells were not readily identified. However when ex[illegible] amining distal portions of the granular tubules with only few granular tubular cells, it coul[illegible] be seen in neighboring sections that both kallikrein and ACA-esterase as well as tryptophan rich substance were present within the same cell (Fig. 2) Moreover practically all cells o[illegible] the granular type were found to contain tryptophan, ACA-esterase, as well as kallikrein using the most suitable fixative for the three *i.e* Helly's fluid formaldehyde, and formalde hyde containing picric acid respectively By prolonging the incubation period for ACA esterase detection, increased intensity in positive cells was observed, but the number of positive cells remained the same.

In pancreatic tissue sections no ACA-esterase activity was observed The whole section was stained light brown but there was no sign of red precipitate within the cells. Also examining the sections in a Leitz Ortholux microscope with a Ploem-type vertical illu[illegible] minator and interference filters for narrow-band excitation of red fluorescence, which in the submandibular gland improved detection of esterase activity did not reveal any positive reaction in the pancreas.

Quantitation of esterases in the submandibular gland and pancreas The amount of kalli krein in the submandibular gland and pancreas was quantitated by SRID and Bz Arg-OEt esterase activity The results are given in Table I In the pancreas, Bz Arg-OEt-esterases were found as pro-enzymes. Activation of pancreas pro-enzymes yielded somewhat less esterase activity than expected from calculations of kallikrein esterase activity based on SRID quantitation and the specific pancreatic kallikrein Bz Arg-OEt-esterase activity (345 EU/mg, Hojima *et al* 1977 b). However in the submandibular gland trypsin and SBTI showed some inhibitory action, and such inhibitory effect may explain the low values in the pancreas.

Table II The specific Bz-Arg-OEt- and ACA-esterase activity of some purified submandibular gland esterases, and the part of the total gland esterase activity which these enzymes represent.

	Bz-Arg-OEt-esterase activity			ACA-esterase activity		
	EU/mg prot	% of total gland act.	EU/gland	TU/mg prot	Calculated TU/gland	Calculated % of total gland act.
Rat submandibular gland esterases						
Esterase A_1	95[a]	7.3	153	13	21	1.2
Esterase A_2	123[a]	3.6	76	114	41	2.3
Esterase B	304[a]	22.3	469	141	217	12.3
Kallikrein	599[a]	66.7	1 401	122	285	16.2
Other esterases		0				68.0
Total gland act.	7[b]	100	2 101 ± 682		1 762 ± 712	100
Rat urinary kallikrein	815			130		
Trypsin (Difco)	5			1 000		

[a] From Brandtzaeg et al. 1976

[b] From Ørstavik and Gautvik 1977

[c] See also Table I where similar calculations were made for each gland separately

In the pancreas kallikrein exists as a pro-enzyme (Fiedler *et al.* 1970, Hojima *et al.* 1977b). Pro-kallikrein and kallikrein (partially purified) show immunological identity and are not distinguishable with the antiserum employed in the fluorescence study (Proud *et al.* 1977). As kallikrein antigen, but not kallikrein esterase activity could be detected in non-activated tissue homogenates, it is possible that kallikrein detected immunohistochemically actually represents pro-kallikrein. However the effect of tissue fixation and processing for immunohistochemistry on enzyme configuration is not known. The localization of pro-kallikrein can only be ascertained by means of an antiserum specific for pro-kallikrein determinants.

The ACA-esterase reaction observed in the rat submandibular gland and the pancreas was shown not to be specific for one particular enzyme. Kallikrein and esterases A and B all have the ability to hydrolyze the ACA-ester although to a varying degree. However in the submandibular gland 68% and in the pancreas 98% of the total ACA-activity represent unidentified enzymes. Thus, the bulk of ACA-activity may very well represent a trypsin-like enzyme different from the esterases tested. Since in the pancreas, ACA-activity was not significantly influenced by pro-enzyme activation, the(se) unidentified ACA-esterase(s) are probably not trypsin which in the pancreas exists as a pro-enzyme.

Kallikrein and ACA-esterase are in the submandibular gland located within the same granular tubular cells as revealed by serial tissue sections. Moreover the presence of the two enzymes in most granular tubular cells excludes the possibility that this is due to kallikrein ACA-activity as kallikrein makes up only 19% of the total gland ACA-activity. In the pancreas, kallikrein shows a distribution similar to the zymogen granules as visualized by the DMAB-staining. Kallikrein(ogen) and trypsinogen are found in isolated zymogen granules (Bhoola and Dorey 1971). ACA-esterase activity has also been found in the rat

TABLE I Kallikrein (antigen), Bz Arg-OEt and ACA-esterase activity in the submandibular gland ar pancreas. Gland homogenates were treated by trypsin-activation, soybean trypsin inhibitc (SBTI), or autoactivation (see Method). Pro-enzymes were not detected in the submandibul gla d whereas in the pancreas Bz Arg-OEt-esterases were found as pro-enzymes. Pancrea ACA-esterase acti ity was ot altered by gland homogenate activation. ( ) number of glands.

	Submandibular gland				Pancreas			
	Non acti-ated	Trypsin + SBTI	SBTI	Calculated act. representing kallikrein[a]	Non-acti-ated	Auto-acti-vated	Trypsin + SBTI	Calculated ac representing kallikrein[a]
Kallikrein (SRID) µg/g w w	11 980 ± 2 253 (14)	n.d.	n.d.		13 ± 51 (6)	n.d.	n.d.	
Bz-Arg-OEt est. act. EU/g w w	11 859 ± 619 (5)	2 697 ± 875 (5)	4 877 ± 6.4 (5)	7 012 ± 962 (5)	0 (6)	40.6 ± 6 5 (5)	16.1 ± .9 (6)	44 9 ± 20 5 (6)
ACA est. act. TU/g w w	8 120 ± 3 422 (14)	n.d.	n.d.	1 432 ± 190 (14) [19 6 ± 6.0 of total act.][b]	1 363 ± 778 (5)	1 003 ± 650 (6)	1 820 ± 979 (4)	16 4 ± 7.5 (6) [1.8 ± 0.4 of total act.

[a] Gland kall krein concentrations were obtained by SRID (see methods). Specific Bz-Arg-OEt-ester activity of submandibular gland k likrein (599 EU/mg) and pancreatic kallikrein (345 EU/mg) has bee given by Brandtzaeg et al (1976) and by Hojima et al (1977 b) respectively Specific ACA-esterase activi of submandibular gland kallikrei is given in Table II For pancreatic kallikrein the mean of submandibula gland and urinary kallikrein specific ACA-activity (122 and 130 TU/mg protein) was used as it was assume that pancreatic kallikrein did not differ much from these t o.

[b] The kallikrein percentage of total ACA-esterase acti ty could also be calculated by kallikrein Bz-Arg OEt-quantitations as 67% of total Bz Arg-OEt-esterase activity is due to kallikrein (Brandtzaeg et a 1976). This gave a kallikrei percentage of total ACA-acti ty of 18.4 ± 2.4 which is equal to that calcu lated from the SRID-kallikrein concentrations.

gland (Ørstavik and Gautvik 1977 Ørstavik et al 1977). Also in the cat submandibula gland immunofluorescence studies demonstrated kallikrein in the duct system (Hojima et al 1977 a). The localization obtained in the present study was controlled by absorbing the fluorescenct conjugate with purified kallikrein from urine and the submandibular gland The identity of these two antigens was established by their kinin-generating ability then oxytocic as well as hypotensive activity and the esterase activity compared to the caseinolytic activity (Nustad and Pierce 1974 Brandtzaeg et al 1976) Moreover antisera producec against these two different kallikreins both gave the same cellular localization of pancreatic kallikrein, i.e in the acinar cells. Despite the similarity in amino acid composition between pancreatic kallikrein and trypsin (Fiedler 1976), these two enzymes (partially purified) did not show immunological cross-reactivity when reacted in double diffusion or in a radio-immunoassay with the same antiserum against rat submandibular gland kallikrein as used in the present study (Proud et al 1977) The presence of trypsin in the acinar cells should therefore not disturb immunohistochemical detection of pancreatic kallikrein. Moreover when the antiserum was reacted against crude pancreas homogenate, only one precipitin line could be detected, and this antigen was immunologically identical to the partially puri-fied kallikrein (Proud et al 1977). Furthermore, absorption of the conjugate with cross-reacting antigens from the submandibular gland did not influence the fluorescence reaction.

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Esterase B	304[a]	22.3	469	141	17	12.3
Kallikrein	599[a]	66.7	1 401	122	715	16
Other esterases		0				68.0
Total gland act.	7[b]	100	2 101 ± 68		1 76[illegible] 71	100
Rat urinary kallikrein	815			130		
Trypsin (Difco)	5			1 000		

[a] From Brandtzaeg et al 1976
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In the pancreas kallikrein exists as a pro-enzyme (Fiedler *et al* 1970, Hojima *et al* 1977). Pro-kallikrein and kallikrein (partially purified) show immunological identity and are not distinguishable with the antiserum employed in the fluorescence study (Proud *et al.* 1977). As kallikrein antigen, but not kallikrein esterase activity could be detected in non-activated tissue homogenates, it is possible that kallikrein detected immunohistochemically mainly represents pro-kallikrein. However the effect of tissue fixation and processing for immunohistochemistry on enzyme configuration is not known. The localization of pro-kallikrein can only be ascertained by means of an antiserum specific for pro-kallikrein determinants.

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[a] From Brandtzaeg et al. 1976

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pancreatic zymogen granules (Lagunoff *et al* 1962) and in the acinar cells of the human pancreas (Hopsu and Glenner 1963) However we did not succeed in demonstrating ACA activity in the rat pancreatic tissue sections. Since in the rat, the pancreas contains only about 1/6 per g wet weight of the submandibular gland ACA-activity the negative result may be due to a concentration below detection limit in the pancreas. The *in vivo* formaldehyde fixation of the pancreas was rapid and complete, therefore autolytic destruction of zymogen granules probably was not the reason for the negative results, even though rapid fixation was critical for kallikrein detection. However in spite of the failure to demonstrate the cellular distribution of ACA-esterase activity in the present study it seems reasonable to assume that kallikrein as well as other trypsin like enzymes, like the ACA-esterase, are located in the acinar cells of the pancreas as they are in the granular tubular cells in the rat submandibular gland.

In the major salivary glands, kallikrein is found within the duct system, and in the kidney in the distal tubules which resemble the striated duct of the salivary glands. In the exorbital lacrimal gland no kallikrein was detected (Ørstavik 1978) and this gland like the pancreas is devoid of striated duct-like structures. Thus the location of kallikrein in the pancreas is different from that seen in other exocrine organs investigated However the acinar cells of the pancreas resemble the granular tubular cells of the rat submandibular gland both have ample amounts of secretory granules containing enzymes like kallikrein and other trypsin like esterases which upon cellular degranulation are secreted into the duct lumen. The physiological importance and the site of action of kallikrein remain unresolved questions. However the presence of kallikrein in the pancreas solely as a pro-enzyme is characteristic for this organ Pro-kallikrein probably is activated by trypsin which itself is activated mainly in the duodenum Therefore, it seems as if structures in the gut may be the location for pancreatic kallikrein function. Functional analyses of kallikrein may thus be facilitated by comparisons of kallikrein action in the duodenum, the striated ducts of the salivary glands, and the distal tubules of the kidney

We are grateful to Dr Kjell Nustad for the supply of purified submandibular gland esterases, urinary kallikrein and antisera against kallikrein. We also want to thank *Dept of Oral Pathol Univ of Oslo* for sectioning of tissue The study was supported by *The Norwegian Research Council for Science and the Humanities.*

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Acta physiol. scand 1978. 103. 394–403
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Attraction of nerve fiber outgrowth from sympathetic ganglia to heart auricles in tissue culture

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Received 15 January 1978

Abstract

ERÄNKÖ O and T LAHTINEN. *Attraction of nerve fiber outgrowth from sympathetic gang... to heart auricles in tissue culture* Acta physiol. scand. 1978. *103* 394–403

Sympathetic chain ganglia of 3-day-old rats were cultured in Rose chambers for 5 days. Each chamb... contained either two ganglia or a ganglion and a piece of heart atrium. Visual examination failed to sh... an increased density or length of nerve fibers growing towards the other explant. However actual measure... ments showed that the length of nerve fiber outgrowth towards the atrium explant was about 10 ... long... than the mean outgrowth to all directions, the difference being statistically significant ($P<0.001$) ... ganglion–ganglion pair cultures the nerve fiber outgrowth towards the other ganglion tended to be short... than that to other directions. It is tentatively concluded that atrium explants exert a growth-promoti... influence on sympathetic nerve fibers in cultures, possibly due to diffusion of growth-promoting substan... from the atrium expla... t

In a thorough discussion of growth and maturation of neurons, Jacobson (1970) pointe... out that direct evidence of chemotactic guidance of nerve fiber growth is not yet availabl... However it has recently been reported by Chamley *et al* (1973) that more nerve fibers fro... explants of rat sympathetic ganglia *in vitro* grow preferentially to nearby explants of normall... densely innervated autonomic effectors, such as the heart and vas deferens, as compare... with fiber growth to explants of normally sparsely innervated organs, such as the kidney... uterus, ureter or lung. In a subsequent study Chamley and Dowel (1975) reported, more... over that sympathetic nerves can distinguish between different parts of the same orga... with different densities of innervation *in vitro* nerve fibers from explants of cultured sym... pathetic ganglia grew mainly to atrium explants, although ventricle explants were presen... at the same distance.

Chamley *et al* (1973) and Chamley and Dowel (1973) based their observations on visua... assessment of nerve fiber growth without any measurements. Since our own visual observa... tions on joint cultures of sympathetic ganglia and target organs failed to show any clea... preferential growth towards densely innervated organs, we decided to carry out a quantita... tive study in which visual examination was supplemented by quantitative measurements o...

the length of nerve fiber growth to different directions from the gangl on explant. Such measurements show without ambiguity how far fibers grow from the ganglion explant toward the target, as compared with other directions. Moreover they make possible the use of statistical methods to assess the significance of the directional growth, if this seems to be present.

Materials and methods

Culture method

Thoracic sympathetic and lumbar [illegible] ganglia and small pieces of heart atrium of about 3-day-old Sprague Dawley rats were cultured in Rose (1954) chambers. The culture method has been described in previous papers (Eränkö *et al.* 1972 a, b). It is the same as that used by Chamley *et al.* (1973) and Chamley and Dowel (1975), except for the mode of arrangement of the explants on the coverslip to be described below.

After aseptic removal under binocular microscope the sympathetic chain [illegible] dissected in [illegible] balanced salt solution (Hanks and Wallace 1949) with 10 [illegible] (v/v) fetal calf serum and 100 000 [illegible] penicillin sodium G. Before culture, the ganglia were treated with 0.125 [illegible] trypsin for 30 min at 37°C, and rinsed in the balanced salt solution. The atrium explants and the ganglia were then placed on coverslips previously coated with collagen (Ehrmann and Gey 1956, Hauschka and Koenigsberg 1966) and covered with [illegible] strip of [illegible] (Rose *et al.* 1958). The chambers were supplied with Medium 199 (Salk *et al.* 1954) supplemented with 20% (v/v) fetal calf serum, 50 [illegible] insulin, 100 000 [illegible] penicillin sodium G and [illegible] glucose. No nerve growth factor [illegible] used. The chambers were kept at 37°C in [illegible] incubator and the pH of the medium was set at 7.2 by [illegible] constant flow of 5 [illegible] carbon dioxide in [illegible] bubbled through water.

Arrangement of explants in chambers

[illegible] Chamley *et al.* (1973) cultured 2 rows of different organs on both sides of [illegible] row of ganglia, [illegible] only 2 explants in each chamber to ascertain the presence or absence of preferential growth towards the other explant. In each chamber [illegible] ganglion was cultured together with another ganglion or piece of heart atrium, about the same size as the ganglion and taken from the same animal. Atrium was selected because this organ was reported by Chamley *et al.* (1973) to readily attract nerve fiber growth. The distance between the explants (from margin to margin) was about 1.5–2 mm.

After setting up the culture chambers, these were left for 5 days undisturbed in the incubator so as [illegible] and [illegible] of possible chemotactic gradients forming between the explants. During this time the [illegible] was horizontal, the explants above it.

Measurement of nerve fiber growth

After [illegible] days the cultures were examined under [illegible] phase contrast microscope and the extent of fiber growth from the explant was measured in 4 perpendicular directions by moving the specimen with the aid of the microscope stage and reading the Vernier scales. These measurements were carried out at [illegible] 125 magnification [illegible] 10, N.A. 0.40) by which the fine nerve fibers were well visible. The outlines of the explants and the outgrowth around them were also drawn with the aid of [illegible] drawing tube and dark field illumination.

Results

General observations

A rich network of nerve fibers always grew out of the ganglion explant, as is shown at low power in Fig. 1. Under the conditions of the present study nerve fibers and spindle shaped glial cells grew together. Thus, neither naked nerve fibers without glial cells nor glial cells without nerve fibers were seen outside the outgrowth border which they together formed, with rare exceptions. This is illustrated in Fig. 2, which shows typical phase contrast photomicrographs from the periphery of the ganglion outgrowth. Nerve fibers and glial cells were readily recognized at the magnification employed for the measurements.



Fig. 1 Low power dark field photomicrograph f a "fused" 5-day ganglion and atrium joint culture A dense network of nerve fib and supporting cells grows out of the ganglion explant in the lower part of the f gure, this outgrowth extends beyond the he trum explant in the upper part of the figu Groups of migrating nerve cells ha e beco detached from the explant and form pe sula and an isla d bove it. M gnificati 20.

As a rule, no outgrowth occurred from the atrium explants. In the rare occasions wh this happened there was no difficulty in discriminating this outgrowth from the nerve fib outgrowth of the ganglion Therefore, it was possible to measure the length of nerve fib outgrowth towards the atrium explant even when the nerve fibers actually grew up to th atrium explant and past it.

If the nerve fibers from the ganglia in pair cultures were long enough so that they met an intermingled between the explants, it was impossible to measure the distance of outgrow to that direction

Nerve fiber outgrowth to different directions

In Fig. 3 are superimposed several drawings made of 5 day cultures, showing the ganglio explant in the center the atrium explant in the periphery and the outlines of the fiber out growth around the ganglion A circle is drawn around the center of the ganglion explant th mean distance to the center of the atrium explant forming a radius. It is obvious from th drawing that the distance of outgrowth around the ganglion greatly varied from one explar to another The outgrowth towards the atrium explant marked 0° is approximately simila to that in any other direction, of which 90° 180° and 270° have been indicated becaus

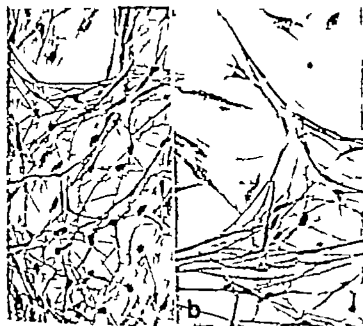


Fig. Phase contrast photomicrographs of peripheral area of outgrowth from a ganglion explant cultured for 3 days. a) Fine nerve fibers accompanied by spindle shaped glial cells form typical dense network. b) Peripheral border of the nerve fiber outgrowth. A growth cone can be seen in the upper left part of the figure. The nerve fibers are closely associated with glial cells. Flat supporting cells, which have the net of nerve fibers and glial cells, can easily be differentiated from these. Objective 10, N.A. 0.40. Magnification 160.

total outgrowth measurements were carried out in these directions. It can also be seen from Fig. 3 that the nerve fiber outgrowth in many cases reached the atrium explant.

Visual examination of the cultures failed to show that the nerve fibers growing towards the other explant, atrium or another ganglion, had been longer or more numerous than those growing to other directions.

Absolute nerve fiber outgrowth measurement

Table I shows the distance of nerve fiber outgrowth in mm to the 4 perpendicular directions illustrated in Fig. 3. The distance of outgrowth was measured both from the center of the ganglion explant and from the border of the explant. The whole material is shown in the upper third of Table I, in the center third are the "fused" cultures, whose outgrowth contacted the other explant or the outgrowth from it, and in the lower third are the "separate" cultures, whose outgrowth did not reach the other explant or its outgrowth.

Because of intermingling of nerve fibers between paired ganglion cultures, it was not possible to measure the extent of growth at direction of 0° in all cultures, and the mean for all cultures is omitted, since it is not representative of the whole material. The upper data will serve to show that the extent of nerve fiber outgrowth to directions 90°, 180° or 270° in ganglion and atrium pair cultures was closely similar to that in paired ganglion cultures. The mean distance of outgrowth from the center of explant towards the atrium



Fig. 1 Low power dark field photomic graph of "fused" 5-day ganglion and atri joint culture. A dense network of nerve fib and supporting cells grows out of the gangl explant in the lower part of the figure, a this outgrowth extends beyond the he atrium explant in the upper part of the fig. Groups of migrating nerve cells have beco detached from the explant and form a pen sula and an island above it. M gnificatn 20.

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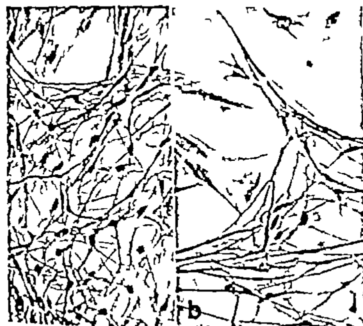


Fig. 2. Phase contrast photomicrographs of peripheral areas of outgrowth from ganglion explant cultured for 3 days. a) Fine nerve fibers accompanied by spindle shaped glial cells form typical dense network. b) Peripheral border of the nerve fiber outgrowth. A growth cone can be seen in the upper left part of the figure. The nerve fibers are closely associated with glial cells. Flat supporting cells, visible above the network of nerve fibers and glial cells, can easily be differentiated from these. Objective 10, N.A. 0.40. Magnification 160.

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Nerve fiber outgrowth measurements

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Table I. Nerve fiber outgrowth (mm) from sympathetic ganglion explant towards another explant (0°) and towards other directions in 5 day pair cultures.

Paired organs	Number of cultured ganglia	Direction of growth measurement (°)[a]	Distance of outgrowth from center of explant (mm) Mean	S.D.	Distance of outgrowth from border of explant (mm) Mean	S.D.
Ganglion and Ganglion (All)	28	0	Not measured[b]		Not measured	
		90	1.3	0.46	1.03	0.45
		180	1.38	0.37	1.10	0.36
		270	1.33	0.48	1.19	0.55
Ganglion and Atrium (All)	54	0	1.64	0.40	1.40	0.45
		90	1.4[illegible]	0.34	1.19	0.34
		180	1.38	0.30	1.18	0.44
		270	1.37	0.36	1.18	0.47
Ganglion and Ganglion (Fused)	12	0	Not measured[b]		Not measured[b]	
		90	1.45	0.33	1.10	0.26
		180	1.58	0.41	1.31	0.28
		270	1.46	0.51	1.19	0.33
Ganglion and Atrium (Fused)	22	0	1.87	0.77	1.66	0.37
		90	1.57	0.28	1.35	0.36
		180	1.48	0.28	1.35	0.50
		270	1.54	0.28	1.41	0.46
Ganglion and Ganglion (Separate)	16	0	0.96	0.51	0.81	0.55
		90	1.23	0.53	0.97	0.56
		180	1.23	0.25	0.94	0.34
		270	1.[illegible]4	0.46	1.11	0.63
Ganglion and Atrium (Separate)	32	0	1.48	0.40	1.22	0.41
		90	1.32	0.33	1.09	0.33
		180	1.3[illegible]	0.29	1.06	0.35
		270	1.25	0.36	1.0[illegible]	0.40

[a] Direction towards the other explant is taken 0°. Other values refer to the clockwise angle (°) against this [illegible].
[b] The fiber outgrowth fused between the two ganglia, the outgrowth in this direction could not be measured.

To study directional growth only the general variation of growth has been eliminated by [illegible], in each ganglion, the extent of nerve fiber outgrowth as percent of the mean [illegible] distance of nerve fiber growth at the four perpendicular directions (Table II). For [illegible] explained above, the relative values could only be calculated in such ganglion pair [illegible] whose nerve fiber outgrowth did not fuse, while the relative growth data for ganglion [illegible] atrium pairs were included whether the growth reached the atrium or not.

Had the outgrowth been equidistant to all directions, the mean relative outgrowth values [illegible] been about 100% to all directions. However in ganglion and atrium pair cultures the [illegible] relative outgrowth of nerve fibers towards the atrium (0°) was over 100% in the whole [illegible] together in "fused" cultures, and in "separate" cultures whose nerve fiber out[illegible] had not reached the other explant or outgrowth from it (Table II). In all 3 instances [illegible] difference was statistically highly significant ($P < 0.0001$), testing with the aid of the t [illegible] the significance of the difference from 100%, a method which best indicates whether [illegible] directional growth had occurred. While the mean outgrowth at 0° increased over 100%,

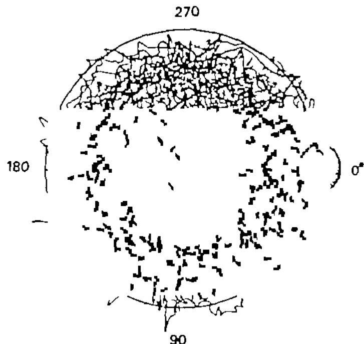


Fig. 3 Outlines of nerve fiber outgrowth around superimposed ganglion explants after culture for 5 d
The ganglion explant is on the left nd the atrium explant on the right. A circle has been drawn around
ganglion explant, the distance between the explant centers as a radius. Note the lmost round outgrow
pattern around the gangl on explant. The length of nerve f ber outgrowth was measured at the 4 perp
dicular directions indicated by n mbers.

(1 64 mm) was larger than that to any of the other 3 directions (1 37–1 42 mm) and th difference was in all instances statistically significant ($P < 0\ 005$) When measured from t border of explant, essentially similar results were obtained but the mean outgrowth at (1 40 mm) was different from that to 90 180° 270 (1 18–1 19 mm) at a less significant lev ($P < 0\ 02$).

For those ganglion pair cultures whose outgrowth zones had fused between gangl (middle third of Table I) the data of outgrowth at 0° were omitted for the same reason above. A slightly longer mean growth towards the atrium, as compared with growth other directions, can be seen in those cultures in which the nerve fibers reached the atriu explant

Data on the "separate" cultures, whose outgrowth did not meet the opposite explant outgrowth from it (lower third of Table I) failed to show consistent, statistically significa differences between 0° and the other directions of outgrowth in either pair group. It shoul be noted that the outgrowths to different directions can only be compared within, not be tween, the two "separate" culture groups, because all those cultures were excluded fro the ganglion pair group whose nerve fibers met between the two ganglia, while only thos ganglia were rejected from the ganglion and atrium pair group whose nerve fibers ha reached the much longer distance to the atrium explant.

Relative distance of nerve fiber outgrowth

The absolute outgrowth data (mm) given in Table I include the large variations in the genera nerve fiber outgrowth from one ganglion to another not only in the direction of growth

area of explant equally applies to the relative outgrowth as measured from the border of explant, which also showed a significant increase in the relative growth towards the atrium and a significant decrease in the relative growth towards another ganglion.

Discussion

Our observations failed to confirm those reported by Chamley *et al.* (1973) and Chamley and Dowel (1975), who found by mere visual examination that many more nerve fibers grew from sympathetic ganglion explants towards normally densely innervated tissues in tissue culture, such as the heart atrium, than away from it or towards explants of such tissues that are normally sparsely innervated. In our cultures, a dense network of nerve fibers regularly grew to all directions around the explant and visual examination failed to show any distinct tendency of preferential growth to any direction. This equally applied to cultures with two ganglia and those with ganglion and atrium explants. No difference was in this respect observed between "fused" cultures, whose outgrowth met in the middle of the ganglion explants or reached the atrium explant, and "separate" cultures, in which the outgrowth from the ganglion explant failed to reach the atrium or the outgrowth of the other ganglion.

It is not easy to explain this discrepancy especially since our culture technique was supposedly identical with that employed by Chamley *et al.* (1973) and Chamley and Dowel (1975), the method having been used by one of us (see Eränkö *et al.* 1972 a, b) in the same laboratory where Chamley *et al.* (1973) and Chamley and Dowel (1975) worked. However there may yet have been small but significant differences. Indeed, while we cultured only 2 explants in each chamber to be able to judge with less ambiguity the eventual growth promoting effect, Chamley *et al.* (1973) and Chamley and Dowel (1975) used 3 rows of explants, ganglion explants in the center row and 2 rows of 5 explants of peripheral organs on both sides of them. Comparison of their photomicrograph (Fig. 4 in Chamley *et al.* 1973) with ours (Fig. 1) suggests that the nerve fiber growth around our ganglion explants was much more profuse than that in theirs. If growth of nerve fibers was in their cultures limited for some reason, diffusion of any growth promoting substances from nearby explants can be expected to have a better chance to stimulate it.

Our data were obtained by recording the coordinates on the Vernier scale of the microscope stage and by drawing the outlines of the ganglia and the nerve fiber outgrowth from them. Since nerve fiber outgrowth was always closely accompanied by glial cell outgrowth, there was no difficulty in discrimination between outgrowth of nerve fibers and supporting cells. Measuring the coordinates of the center of the ganglion explant can be thought to be subject to unconscious bias, and it is therefore important to note that similar results were obtained when measurements were carried out from the border of the explant.

In view of the subjective impression first obtained by mere visual examination that the nerve fiber outgrowth was about the same to all directions, the results of our quantitative measurements were unexpected in showing at a high level of statistical significance that nerve fibers grew farther towards the atrium, as compared with the other directions. This result was obtained for the whole material together as well as for the "fused" and "separate"

TABLE II Relative extent of nerve fiber outgrowth as percent of mean growth towards 4 perpendic[illegible] directions in 5 day pair cultures. After culture for 5 days, the distance of outgrowth was meas[illegible] towards the other explant and towards the 3 other perpendicular directions. In each pair cul[illegible] the mean of these 4 values was taken as 100% and the outgrowth towards the 4 directions [illegible] expressed as % of the mean growth, thus equalizing variations between cultures in the ger[illegible] growth.

Paired organs	Number of cultured ganglia	Direction of growth measurement (°)[a]	Relative outgrowth from center of explant (%) Mean	S.D	Relative outgrowth from border of explant (%) Mean	S.D
Ganglion and Atrium (All)	54	0	112.57[b]	13.41	113.57[b]	17.81
		90	95.00	13.12	97.28	16.73
		180	96.06	11.94	95.72	19.46
		270	94.11	13.73	94.15	21.01
Ganglion and Atrium (Fused)	22	0	116.23[b]	9.42	116.50[b]	15.40
		90	97.86	10.17	93.55	12.09
		180	92.00	12.28	93.50	17.58
		270	95.86	10.36	96.95	16.12
Ganglion and Ganglion (Separate)	16	0	76.13[b]	32.24	77.88[b]	35.66
		90	101.38	35.24	97.56	47.34
		180	111.25	28.97	105.38	31.60
		270	111.38	52.12	119.94	6[illegible]
Ganglion and Atrium (Separate)	32	0	110.06[b]	15.21	111.56[b]	1[illegible]
		90	98.09	14.98	99.84	1[illegible]
		180	98.84	11.04	97.25	2[illegible]
		70	92.91	15.68	92.22	2[illegible]

[a] Direction towards the other explant is taken as 0. Other values refer to the clockwise ang[illegible] this line.
[b] This value differs significantly from 100% at the level P < 0.0001

the outgrowth at the other 3 directions correspondingly decreased below 100% [illegible] and atrium pair cultures, whether the whole material was considered together and "separate" cultures distinctly. However this mean decrease in relative growt[illegible] direction was not always statistically significant because the difference fron[illegible] 100% at 90° 180° and 270° was less than that at 0° while the standard deviatio[illegible] the same. This should not detract from the main point that the relative outgro[illegible] the atrium was significantly larger than that to the other three directions.

Fused" ganglion and atrium cultures showed a larger relative outgrowth [illegible] atrium (116.23%) than did "separate" cultures of ganglion and atrium (110.0[illegible] this difference was not statistically significant, a highly significant difference b[illegible] two groups was observed, when only such cultures were considered in whic[illegible] distance was 1.5 mm.

In the "separate" ganglion pair cultures the outgrowth towards the other [illegible] 76.13%, *i.e.* much less than that to the other 3 directions. This result can als[illegible] by saying that the relative distance of growth towards the other ganglion was [illegible] than the mean growth, 100%. This difference was statistically highly significant (P < 0.000[illegible]

All what has above been said concerning the relative nerve fiber outgrowth from th[illegible]

[illegible], it is too early to speculate on the nature of such postulated substance. Nerve growth factor was proposed by Chamley *et al* (1973) but diffusion of a less specific substance [illegible] from the atrium may have been responsible, many of which are known to have pronounced effects on the growth and metabolic abilities of nerve cells (Murray 1976, [illegible] *et al* 1976)

It is even more open to doubt whether release of a specific substance from the target organ [illegible] guide to it the nerve fibers from the perikaryon in a developing living animal. However the problem is fascinating and continued efforts to sol e problems of directional nerve fiber growth can be expected to furnish significant new observations.

The expert technical assistance in tissue culture by Mrs Marja Järvinen is gratefully acknowledged. M [illegible] skilfully prepared the photographic prints.
This work was supported by grant from the Sigrid Jusélius Foundation to Ola Eränkö.

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cultures separately whether the measurements were made from the center or the border of the ganglion explant. We conclude therefore that there was a real though small, increase *in the mean length of those nerve fibers that grew towards* the atrium.

Another unexpected result that emerged from the measurements was the highly significant decrease in the length *of nerve fibers growing towards the other ganglion explant in "separate"* ganglion pair cultures, as compared with growth to other directions. However care is necessary in interpreting this observation because *the length of growth towards the* other ganglion could only be measured in the "separate" ganglion pair cultures whose outgrowth had not fused in the center of the explants, the "fused" ganglion pair cultures having purposely been excluded It is therefore possible that the shorter mean fiber growth towards the other ganglion was due to selective exclusion of such ganglion pair cultures whose fibers grew better towards the other ganglion. This matter should be re-examined using shorter culture times or a longer distance between the ganglion explants. A similar selection mechanism may have been responsible for the observed tendency towards longer fibers growing towards the atrium in "fused" ganglion–atrium cultures, as compared with "separate" cultures. However it cannot be excluded that the nerve fibers growing towards the atrium were truly longer because they had contacted the target tissue. Further studies are necessary to elucidate also this point. On the other hand, since the "separate" ganglion–atrium culture group was formed by selecting ganglia whose nerve fibers were *short* enough not to reach *the atrium, it is even more significant that the nerve fibers growing towards the atrium were* yet significantly *longer* than those growing to other directions.

Our main observation is, then, that nerve fibers from the sympathetic ganglion growing towards the atrium were a little, but highly significantly longer than those growing to other directions. The conclusion must be drawn from this observation that the atrium explant somehow stimulates the nerve fiber growth, a conclusion similar to that drawn by Chamley *et al* (1973) and Chamley and Dowel (1975) as different as their results otherwise were from ours.

The existence of such growth promotion has long been suspected Since nerve fibers from the proximal stump of a divided nerve tend to enter the peripheral stump Forssman (1900) proposed that "neurotropism" is responsible, and Ramon y Cajal (1910) thought that nerve fibers might indeed be attracted by chemicals released from tissues. Harrison (1910) formulated the problem as follows "If it could be shown in tissue culture that there is an attraction between growing nerve fibers taken from a certain part of the nervous system and a particular kind of peripheral cell then we should have direct evidence for the existence of those more subtle factors which seem to be necessary to account for the definitive establishment of particular nervous connections." However Harrison's (1910) own experiments of this kind gave negative results, and Jacobson (1970) more recently pointed out that "successful experiments of the type that Harrison suggested more than 50 years ago have not yet been made"

In view of these authoritative statements, any evidence of nerve fiber growth stimulation exerted by the target tissue can be considered useful. It seems possible that the atrium explant may have released a substance promoting nerve fiber growth and that a concentration gradient of this substance was formed from the atrium explant to the ganglion explant.

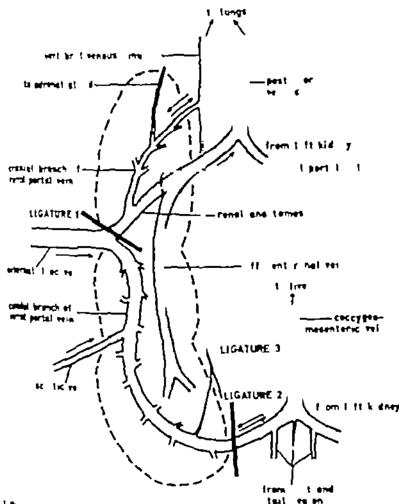


Fig. 1. Principal veins of the avian renal portal system of the right kidney seen from the abdominal cavity. Venous blood from the leg enters the renal portal vein via the external iliac and the sciatic veins, and constitutes the main afferent flow to the renal portal system of intact hens (Odlind 1978). This blood can either perfuse the peritubular capillaries of the kidney (bypassing the glomeruli) or be shunted 1. to the lungs via the renal anastomoses and/or via the cranial branch of the renal portal vein or 2. to the liver via the coccygeomesenteric vein. For further details see Odlind (1978). Ligatures 1–3 (see text) are placed round vessels at points indicated by crossmarks.

From the foregoing, however, it seems that ideal conditions for use of the Sperber technique would be created if the shunting of renal portal blood could be abolished. It has already been shown (Odlind 1978) that, by ligating the appropriate shunt vessels (Fig. 1) all portal blood from the leg can be forced to perfuse the kidney as determined shortly after the operation. In the present study it was tested if this renal perfusion of all portal blood from the leg persisted, so that the true tubular excretion fraction (TTEF, Sperber 1948 b), a measure of the tubular excretion efficiency of a substance, could be estimated directly. Since this was found to be the case, it is concluded that excretion studies in ligated animals offer a valuable complement to the original Sperber technique.

Acta physiol. scand. 1978 103, 404–412

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A modified Sperber technique for direct estimation of true renal tubular excretion fraction

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Received 16 January 1978

Abstract

Odlind, B. *A modified Sperber technique for direct estimation of true renal tubular excretion fraction.* Acta physiol. scand. 1978, *103*, 404–412.

The Sperber technique in the hen is particularly suitable for the study of renal tubular secretion. However, results obtained with this technique vary considerably due to an unpredictable and highly variable shunting of renal portal blood. In an attempt to get a total and stable renal perfusion by portal blood from the leg, appropriate shunt vessels were ligated. This procedure was found to force all portal blood from the leg to perfuse the ligated kidney without affecting the symmetry of glomerular filtration or renal clearance of 125-I Na-o-iodohippurate between the kidneys. In conclusion, the abolition of renal portal shunt flow allows use of the Sperber technique for a direct estimation of the true tubular excretion fraction (TTEF) of a substance. Thus, $TTEF_{PAH}$, a measure of the tubular excretion efficiency of para-amino-hippuric acid, was found to be about 70%. The stable renal perfusion in ligated animals will also facilitate comparative studies of renal tubular excretion in the hen. Moreover, no animal has to be rejected due to low renal perfusion of portal blood. Furthermore, the use of ligated hens makes it unnecessary to use markers for the renal perfusion of portal blood when steady-state experiments are performed. Finally the total renal perfusion of portal blood in ligated animals will facilitate the demonstration of a secretory component in the renal handling of substances with a low affinity for the renal transport system.

Key words: Renal clearance, tubular secretion, kidney, birds, microspheres

Based on the renal portal circulation in the hen, Sperber (Sperber 1946, 1948 a, b) has developed a technique, which is particularly suitable for the study of renal tubular secretion, cf. reviews by Weiner (1973) and Rennick (1976). However, the results obtained with this technique vary considerably due to an unpredictable and highly variable shunting of renal portal blood to the liver and to the lungs (Odlind 1978). In spite of this variation, individual excretion values may be compared, if the fraction of portal blood that perfuses the kidney is known. This fraction can be determined from the tissue distribution of microspheres injected simultaneously with the substance to be tested (Odlind 1978). An alternative method is to co-administer a marker for the renal perfusion, e.g. para-amino-hippuric acid (Rennick 1976). The excretion efficiency of the test substance can then be expressed relative to that of the marker.

[illegible] tubular excretion fraction of para-amino-hippuric acid ($ATEF_{PAH}$)

[illegible]-hippuric acid (PAH) [illegible] administered either as [illegible] 4 5 min bolus injection of tracer doses of 14C-PAH ([illegible]-14-C, 0 23 mCi [illegible], New England Nuclear USA, 9 hens) or as a continuous infusion [illegible] of the sodium salt (Merck, Sharp and Dohme Co, USA 14 hens) into a leg [illegible] the ligated side. After the bolus injections urine [illegible] as collected for three 10 min periods. [illegible] the [illegible] continuous infusion of PAH, its ATEF [illegible] as calculated during steady-state as the mean of [illegible] values [illegible] collection periods in each hen. PAH [illegible] as determined according to Bratton (1951) and 14-C [illegible] by liquid scintillation counting. ATEF [illegible] as calculated by dividing the excess excreted in the urine [illegible] the ligated side with the amount infused (Sperber 1948 b). The total (ipsilateral plus contralateral) [illegible] of PAH averaged 92.1 [illegible] and 96 4 [illegible] of the given amount after bolus injections [illegible] and continuous [illegible], respectively.

There was no binding of 14-C-PAH (0 1 10 μg/ml buffer) to plasma proteins (pooled, heparinized, [illegible] hen plasma) at 37 C as determined by equilibrium dialysis in isotonic phosphate buffer ([illegible], [illegible] and Odlind-Cederlöf 1975). This is in [illegible] agreement [illegible] with the findings by Sykes (1960). S[illegible] and [illegible] (1978), however found 14 [illegible] protein binding of PAH in hen plasma. This discrepancy [illegible] proportions of the tw[illegible] plasma albumins (McIndoe 1962), in the several breeds.

[illegible] of the renal clearances of 51-Cr-EDTA (C_{EDTA}) *and 125-I-Na-o-iodohippurate* ($C_{[illegible]}$)

To [illegible] whether the ligation of vessels in the renal portal system had any effects on the renal func[illegible], the clearances of 51-Cr-EDTA (ethylene-diamine-tetra-acetate, Behringwerke AG, West Germany; [illegible]) and 125-I-Na-o-iodohippurate (Kabi Diagnostika Sweden, 17 hens) were determined for the ligated and the control kidneys separately as described earlier (Odlind 1978).

[illegible] spheres

[illegible] clearance expt. was followed by an injection of 57-Co or 58-C[illegible] labelled 50 μm microspheres (Tracer [illegible], Pharmacia Fine Chemicals AB, Sweden) into [illegible] leg vein on the ligated side and sometimes also [illegible] the control side, using the dual-isotope technique. This technique as described earlier by Odlind (1978), [illegible] [illegible] estimate of the fractional distribution of blood flow from the external iliac [illegible] vein.

Statistical analysis

[illegible] are given as the arithmetic means ± standard deviation (S D) or the arithmetic means ± standard [illegible] of the mean (S E) [illegible] appropriate. Differences in clearance [illegible] values and ATEF [illegible] values were analysed [illegible] with the t-test for paired and unpaired observations, respectively (Armitage 1973) and [illegible] were considered significant at the 0 05 level.

Results

Effect of ligatures on the distribution of renal portal blood

After injection of microspheres into a leg [illegible] vein on the ligated side, nearly all spheres were recovered in the middle and caudal parts of the ligated kidney at 1 1/2–4 h (7 hens) or 2 days ([illegible] hens) after the operation, see Table I. After 4–7 days (19 hens) the recovery was lower than 80 [illegible] in the same parts of the ligated kidney while the recovery in the lungs was cor[illegible] respondingly higher. These results in ligated animals are in sharp contrast to the highly variable distribution of spheres after leg vein injections in non-ligated animals (Odlind 1978). After injection into a leg vein on the control side 4–7 days after the operation, the spheres were distributed between the ipsilateral kidney and the lungs (Table I). No spheres were found in the liver which shows that the shunt flow [illegible] in the coccygeomesenteric vein from both renal portal systems had been blocked.

Effect of ligatures on the venous pressure in the renal portal system

The ligation procedure gave an immediate increase, about 30 %, of the pressure in the external iliac vein on the ligated side (Table II). This pressure remained elevated throughout

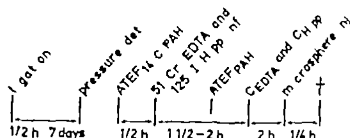


Fig. 2. Experimental schedule. The effects of ligati g vessels in the renal portal system was determine sequence at different times post-operatively using the following parameters. I the venous pressure in external iliac veins, 2. the renal excretion of para-aminohippuric acid ($ATEF_{PAH}$) or 14-C-para-am hippuric acid ($ATEF_{14\text{-}C\text{-}PAH}$), 3 the renal clearances of 51-Cr-EDTA (C_{EDTA}) and 125-I Na-o-iodo purate (C_{Hipp}) and 4 the distribution f 50 μm microspheres injected into leg vein

Materials and Methods

Animals

Laying Rhode Island Red or Kathlin hens, 6–12 months of age and weighing 1 7 to 2 2 kg were used. Th were kept on a commercial feed for laying hens (Pullfo Lantmännen, Sweden) but were fasted over ni before surgery and clearance experiments. They had free access to tap water except during the experimen

Experimental procedures

Fig. 2 shows the different steps of the investigation. The animals were al ays unanesthetized except dur the operations. They were loosely restrained in a harness of cloth (Campbell 1960 a) and sitting in th normal perching position, unless stated otherwise. Urine was collected through two plastic funnels se one over each ureteral opening according to a modification (Campbell 1960 a) of Sperber' original te nique (1946, 1948 a). To prevent clogging by urinary precipitates, each funnel as perfused ith distill water by an nfusion pump at a rate of 1.0 ml/min. The diluted urine was collected in plastic vials on fr tion collector (10 min period), and the volumes determined by weight. Systemic infusions ere ma into a brachial vein and renal portal infusions (injections) into the right medial tarsal ein, unless stat otherwise. Polyethylene catheters (PP50) were used. Isotonic saline was i fused at a rate of 0.15 ml/m throughout the experiments.

Ligation of shunt in the renal portal system

Ligatures were places around the renal anastomosis (ligature 1 in Fig. 1) and the caudal branch of the ren portal vein, where the vessel leaves the kidney (ligature 2 in F g. 1), as described earlier (Odlind 197 This was always done on the right kidney and should force all enous blood from the right leg from th external iliac nd sciatic veins, to perfuse the peritubula capillaries of the middle and caudal parts of th kidney Ideally the same proced re should be performed also on the left side, but here the caudal shun vessel is not accessible to l gation, since it is covered by the dorsal ligament of the oviduct. Instead, a attempt was made to improve the symmetry total renal blood flow between the kidneys by ligating th coccygeomesenteric vein in its caudal 1/3 (ligature 3 in Fig. 1) This should convey the venous blood fro the pelvis, the cloaca nd the coprodeum to the left renal portal system. Moreover it should block th shunting of renal portal blood from the left side to the liver The surgery wa performed without asept precautions, but no infections were seen in bout 50 hens. Acute operation mortality was about 10 mostly due to bleeding. About 5% of the animals were rejected because of complications, *e.g.* stricture o the right ureter

Measurement of venous pressure in the renal portal system

In three animals heparinized polyethylene catheters (PP50) were inserted into the medial tarsal ein o both legs, with the tip of the catheters in the external iliac veins. Venous pressure was then determined o both sides using a diaphragm pressure transducer (type 4-327 L221 Bell and Howell Ltd, England) with the unanesthetized animal supine a well as sitting in the normal perching position. Ligation procedures were then performed (see above) and the pressure determinations repeated soon after the operation and at 24 h intervals thereafter In seven other animals the pressure determinations were done only once, 4–7 days after the operation.

Table III. Renal clearances in ml/min/kg of 51-Cr-EDTA (C_{EDT}) and 125-I Na-o-iodohippurate (C_{IOHip}) 4-7 days after ligating vessels in the renal portal system (means ± S.D. calculated from means of 2-3 10 min clearance periods during steady-state in each of 18 and 17 hens, respectively). The paired differences between values for the ligated and the control kidney are also shown (mean ± S.D.).

	C_{EDT}	C_{IOHip}
Ligated kidney	1.50 ± 0.35	15.5 ± 4.3
Control kidney	1.55 ± 0.38	15.5 ± 5.1
Mean (ligated-control)	0.05 ± 0.16	−0.02 ± 2.7

[illegible] was determined by dividing the ATEF $_{AR}$ value with the renal perfusion fraction of portal blood from the leg. It should be noticed, however that the TTEF $_{AR}$ values of non-ligated animals may be too low since they derive from injections of PAH on the right side, where the mean renal perfusion fraction is about 3/4 of that on the left (control) side (Odlind 1978).

Effect of ligatures on renal function

The renal clearance of 51-Cr-EDTA (C_{EDTA}) was symmetrical ($p > 0.2$) in ligated animals (Table III). The C_{EDTA} obtained was comparable to that of non-ligated hens of the same lot and determined under similar experimental conditions, 1.43 ± 0.20 ml/min/kg for each side (mean ± S.D. in 6 hens, unpublished observation). Since C_{EDTA} equals the renal clearance of 3H-inulin in the hen (Odlind 1976), this indicates that the ligatures had no effect on the glomerular filtration rate. Moreover the renal clearance of 125-I Na-o-iodohippurate was the same for ligated and control kidneys (Table III $p > 0.9$) indicating symmetry between the kidneys of ligated animals also for this acid which is excreted mainly by tubular secretion. Presumably this indicates symmetry also in total renal plasma flow between the two kidneys.

Discussion

The ligation procedure gave the anticipated results when tested by the microsphere technique shortly and 2 days after the operation, since all spheres were recovered in the middle and the caudal parts of the ligated kidney (Table I). This indicates that the blood from the right leg perfused these parts exclusively. Preventing the leg vein blood from reaching the cranial part of the ligated kidney (Table I) is of little or no importance, since very little of this blood perfuses the same part of the kidney even in intact hens (Odlind 1978).

4-7 days after the operation, about 80 % of the spheres were recovered in the ligated kidney whereas the rest were recovered in the lungs. In spite of this, similar ATEF $_{AR}$ values were obtained at different times after recovery from the anesthesia. Moreover these ATEF$_{PAH}$ values were slightly higher than the true tubular excretion fraction of PAH (TTEF$_{PAH}$) of non-ligated animals. This strongly indicates that all portal blood from the leg had perfused the peritubular capillaries of the ligated kidneys also 4-7 days after the ligation. ATEF values obtained in ligated animals thus represent values for the true tubular excretion fraction (TTEF). The escape of spheres to the lungs at 4-7 days after the ligation is therefore probably best explained by leakage of some spheres through the peri-

TABLE I Distribution in different organs of 50 μm microspheres injected into a leg vein at different ti[illegible] after ligating vessels in the renal portal system. Means ± S.D or means and range (betw[illegible] brackets) are given.

Organ	Injection side, time after ligation and number of animals			
	Ligated side			Control side
	1 1/2–4 h 7	2 days 7	4–7 days 19	4–7 days 8
Ligated kidney				
Total	$98.3 \pm 1\ 6$	95.1 ± 4.5	80.9 ± 16.1	39.5 (5 8–83.1)
Cranial part	0.9 ± 0.5	0.6 ± 0.4	2.1 ± 1.6	
Control kidney	0.3 ± 0.2	0.5 ± 0.8	0.9 ± 0.6	$0\ 7 \pm 0.4$
Lungs	$0.9 \pm 1\ 0$	2.6 ± 2.6	17.5 ± 16.2	59.3 (15.2 94 8)
Liver	0.2 ± 0.1	1.8 ± 2.8	0.6 ± 0.5	0.5 ± 0.3

the whole test period of 7 days. No obvious change was seen on the control side. The press[illegible] fluctuated with the phases of respiration, somewhat less on the ligated side.

Effect of ligatures on the renal excretion of PAH

The apparent tubular excretion fraction of PAH ($ATEF_{PAH}$) was the same whether dete[illegible] mined after bolus injections of 14-C PAH or after continuous infusions of PAH (data n[illegible] shown) Soon 1 1/2–4 h after the ligation, before the animals had recovered from the pent[illegible] barbital anesthesia, the $ATEF_{PAH}$ values were low However at 6 h–2 days and 4–7 da[illegible] after the ligation $ATEF_{PAH}$ were $66.7 \pm 4.3\,\%$ and $71\ 6 \pm 1.9\,\%$ (means ± S.E. in 6 and 2[illegible] hens, respectively: $p > 0.2$) Both values are somewhat higher than the true tubular excretio[illegible] fractions of PAH ($TTEF_{PAH}$) $60\ 1 \pm 1.5\,\%$ and $65\ 1 \pm 2.3\,\%$ in non-ligated hens (Odlin[illegible] 1978 means ± S E. from two consecutive determinations in 9 hens). $TTEF_{PAH}$ (cf Sperbe[illegible]

TABLE II Venous pressure in cm H_2O in the external iliac veins before nd at different times after liga[illegible] vessels in the renal portal system in 10 hens. Results are given as means for each side and a[illegible] means and ra ge for the paired differences between the pressures on the two sides. Asterisk[illegible] indicate consecutive measurements in each of three animals (see Methods).

Side and body position	Before	Soon after	Time after ligation (days)						
			1	2	3	4	5	6	7
Ligated									
Supine	7.2	9.2	10.2	11 0	14 3	11 3	10.0	12 5	15.8
Sitting	11.5	—	12.3	14.3	19.3	18 8	15.0	18.5	18 8
Control									
Supine	7.3	6.3	6.8	5 3	6.0	4 8	6.0	4 0	5 0
Sitting	11.5	—	9 7	10.3	10.8	13.3	8.5	10.5	11.0
Difference (lig.-contr)									
Supine	−0 2 (−0.5–0)	2.9 (2 3.5)	3.3 (2.5–4.5)	5.7 (5–6)	8.3 (3–15.5)	6.5 (1 12)	—	—	10 8 (7.5–14)
Sitting	0	—	2.7 (0–7)	4 0 (1–6)	8.5 (5–15.5)	5.5 (3–9)	—	—	7.8 (5–10.5)
Number of animals	3	3	3	3	3	6	1	1	2

Table II. Renal clearances in ml/min/kg of 51-Cr-EDTA (C_{EDTA}) and 125-I-Na-o-iodohippurate (C_{HIPP}) 4-7 days after ligating vessels in the renal portal system (means ± S.D. calculated from means of 2-3 10 min clearance periods during steady-state in each of 18 and 17 hens, respectively). The paired differences between values for the ligated and the control kidneys are also shown (means ± S.D.).

	C_{EDTA}	C_{HIPP}
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(PAH) was determined by dividing the $ATEF_{PAH}$ value with the renal perfusion fraction of portal blood from the leg. It should be noticed, however that the TTEF $_{PAH}$ values of non-ligated animals may be too low since they derive from injections of PAH on the right side, where the mean renal perfusion fraction is about 3/4 of that on the left (control) side (Odlind 1978).

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4-7 days after the operation, about 80% of the spheres were recovered in the ligated kidney whereas the rest were recovered in the lungs. In spite of this, similar $ATEF_{PAH}$ values were obtained at different times after recovery from the anesthesia. Moreover these $ATEF_{PAH}$ values were slightly higher than the true tubular excretion fraction of PAH ($TTEF_{PAH}$) of non-ligated animals. This strongly indicates that all portal blood from the leg had perfused the peritubular capillaries of the ligated kidneys also 4-7 days after the operation. ATEF values obtained in ligated animals thus represent values for the true tubular excretion fraction (TTEF). The escape of spheres to the lungs at 4-7 days after the operation is therefore probably best explained by leakage of some spheres through the peri-

tubular capillaries of the lower 2/3 of the ligated kidney These capillaries may have been dilated by the persistent increase in renal portal pressure (Table II).

What $ATEF_{PAH}$ value would be expected in non ligated animals? Since the tubular excretion efficiency of PAH is 70 % (see Results section), and the mean renal perfusion fraction of portal blood from the leg is 43 5 % (range 8–88 %), as determined by Odlind (1978) using the microsphere technique, a mean $ATEF_{PAH}$ value of 30 % (range 6–62 %) can be calculated This predicted value agrees with the mean $ATEF_{PAH}$ value of 30 % (range 1–48 %) found by Odlind (1978) Sykes (1960) and Skadhauge (1964) found a similar average $ATEF_{PAH}$ values Other workers, however (*e.g* Child and Dodds 1965 May and Carter 1970 Acara *et al* 1975) have reported higher $ATEF_{PAH}$ values, between 50–80 %. The reason for this discrepancy is not clear but the higher values could be due to the preferential use of animals found to excrete a high ipsilateral excess of a test substance, *e.g* phenol red, as recommended by Sperber (personal communication) and described by Campbell (1960 b). Moreover the fraction of portal blood that perfuses the kidney on the side of the injection may sometimes be larger than that on the control side (Odlind 1978) which would amplify the ATEF value This later source of error could explain the extremely high ATEF values (up to 100 %) sometimes seen (Rennick 1958)

The incomplete tubular excretion of PAH after one passage through the kidney as indicated by a $TTEF_{PAH}$ value of about 70 %, could have several explanations. Firstly some renal portal blood could have perfused areas of the kidney that are unable to extract PAH *e.g* the medulla (see review on the renal handling of PAH in the mammalian kidney by Weiner 1973) However Dencker and Odlind (unpublished observations) using an autoradiographic technique found that 57-Co labelled 15 μm microspheres, injected into a leg vein could be seen only in cortical areas of the hen kidney Moreover a perfusion of the avian medullary tissue by renal portal blood has been considered less likely for anatomical (Sperber 1948 a) and physiological reasons (Skadhauge 1973). Secondly the tubular transport capacity of PAH could have been exceeded but since the doses of PAH and the tracer doses of 14-C PAH gave equivalent ATEF values in ligated animals, this was certainly not so (*cf* Odlind 1978). Thirdly it is possible that the avian renal handling of PAH also involves tubular reabsorption, as has been demonstrated in *Necturus* (Tanner and Kinter 1966) and in several mammalian species (Schnermann and Thurau 1965 Baines *et al* 1968, Cho and Cafruny 1970). Finally it is possible that there is an incomplete cortical extraction of PAH in the hen, as has been shown to be the case in the cat (Nissen 1967) and in the dog (Aukland and Loyning 1969 Velasquez *et al* 1972) To evaluate the last two possibilities further studies are needed. Ligated animals offer an advantage in such studies.

An excretion efficiency of PAH after one passage through the avian kidney of about 70 % means that the renal clearance of this substance (C_{PAH}) will significantly underestimate the total renal plasma flow in birds. Consequently when C_{PAH} is used to calculate the filtration fraction of the avian kidney the value of 7–8 % as reported by Sperber (1960) and Skadhauge (1964) is too high. Pitts (1938) obtained the same value for the filtration fraction using the renal clearance of phenol red. Thus, when trying to estimate the maximum ATEF value that could result from diffusion of a substance through tubular cells into the tubular fluid, as defined by the filtration fraction (Sperber 1948 b), a value below 8 % does not exclude

tubular secretion of the substance (*cf.* Sperber 1948 b, Erne and Sperber 1974). Moreover the theoretical limit value for the "diffusion-ATEF" only applies when all portal blood from the leg perfuses the kidney. This is important, since it has been shown (Odlind 1978) that the kidney is perfused by a submaximal fraction of the portal blood from the leg, giving a proportional reduction in the limit ATEF value. Experimental support of this has been obtained in studies of the renal excretion of 125-I Iothalamate in the non-ligated hen (Odlind 1978). The ATEF value of this substance was 8 ..., a figure which would not demonstrate whether the substance is secreted or not. However, the substance is indeed secreted by the tubules, since its renal clearance was reduced by half and reached the values of the glomerular filtration rate, as measured by 3-H-inulin or 51-Cr EDTA, after inhibiting the anion transport system by novobiocin.

It is essential for a correct interpretation of the ATEF values to know if the two kidneys are symmetrical with respect to glomerular filtration and total renal plasma flow (Sperber 1948 b). Both parameters were symmetrical on an average in ligated animals (Table III) which would justify their use in the Sperber technique, but individual testing for symmetry is still necessary.

Excretion studies in ligated animals offer a valuable complement to the original Sperber technique in several ways. Firstly, the true tubular excretion fraction (TTEF, Sperber 1948 b), a measure of the tubular excretion efficiency of a given substance may be estimated directly. Secondly, the large variation in distribution of renal portal blood from the leg seen in intact animals (Odlind 1978) will be abolished, reducing the intra- and interindividual variations in the excretion values. This reduces the number of experiments needed for a reliable comparison of the tubular excretion efficiency of different substances. Moreover no animal has to be rejected due to a low renal perfusion of portal blood (*cf.* Campbell 1960 b, Skadhauge 1964, Sanner 1965). For the same reasons ligated animals are suitable for steady-state experiments. The stable renal portal blood flow in these animals makes it unnecessary to use markers for the renal perfusion fraction of portal blood from the leg (Rennick 1976, Odlind 1978 a). Finally, the use of ligated animals will facilitate the demonstration of a secretory component in the renal handling of substances with a low affinity for the renal transport system.

The present work was supported by grant no. 2874 from the Swedish Medical Research Council. I am deeply indebted to Mrs Sigrid Sandberg for skilful technical assistance.

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Muscle fatigue and its relation to lactate accumulation and LDH activity in man

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Received 20 January 1978

Abstract

TESCH, P. B. SJÖDIN, A. THORSTENSSON and J. KARLSSON. *Muscle fatigue and its relation to lactate accumulation and LDH activity.* Acta physiol. scand. 1978. *103* 413–420.

The lactate concentration in different muscle fibre types was determined in biopsy specimens from human vastus lateralis muscle after 30 and 60 s of sustained dynamic leg exercise. In addition, muscle fibre type distribution, total lactate dehydrogenase (LDH) activity and isozymes of LDH were determined. In accordance with previous studies (Thorstensson and Karlsson 1976, Nilsson *et al.* 1977) it was found that a high proportion of slow twitch (ST) fibres corresponded to better sustained muscle force. Lactate was found preferentially in fast twitch (FT) fibres after 30 s, but after 60 s this difference was abolished. Differences between the two main muscle fibre types in muscle lactate, total LDH activity and M-LDH activity were correlated to muscle fatigue. It was concluded that lactate or associated pH changes mainly in FT fibres could be one factor responsible for the impaired muscle function.

Key words: Fatigue, isozymes, lactate, lactate dehydrogenase, muscle fibre types

An isokinetic fatigue test, performed as repeated maximal knee extensions at a high angular velocity was recently carried out in order to study muscle fatigue in human beings (Thorstensson and Karlsson 1976). Fatiguability measured as the decline in force with 50 contractions (approximately 60 s), was found to be closely correlated to the relative distribution of fast twitch (FT or type II) muscle fibres in the recruited muscles. Similarly the fact that slow twitch (ST or type I) muscle fibres are more resistant to fatigue than FT muscle fibres has been well-established from animal experiments (Burke and Edgerton 1975).

In a subsequent series of experiments (Nilsson *et al.* 1977) EMG recordings were included to investigate if the decline in muscle performance was due to factors located in the neuromuscular junction, as indicated by Stephens and Taylor (1972) or in the muscle fibres as suggested by e.g. Merton (1954). No changes in the EMG activity with fatigue could be demonstrated and it was therefore concluded that local factors in the muscle fibres (mainly FT) were responsible for the impaired muscle function.

Lactate accumulation in the muscle has often been discussed as one inhibitory factor in

relation to muscle fatigue (Karlsson 1971 Simonson 1971). Tesch *et al* (1978) rec demonstrated a relationship between muscle lactate accumulation and the percentage o fibres after 25 maximal voluntary contractions, using the same experimental set-up above, indicating a higher lactate production in FT fibres than in ST fibres. Higher act of the lactate forming enzyme, lactate dehydrogenase (LDH), and a LDH isozyme pat more in favour of lactate production have also been demonstrated in FT as compare ST fibres (Sjödin 1976, Thorstensson *et al* 1977)

The question arises as to whether lactate accumulation changes within recruited mu fibres are responsible for the impaired muscular function and fatigue. For that reason present investigation was undertaken to study changes in lactate concentration in the main muscle fibre types during different stages of the fatigue test and to relate tl differences to LDH activity and isozyme pattern in the different fibre types.

Subjects, Methods, and Procedure

9 male physical education students participated in the study. Their mean (±S.E.) age, height, weight, $\dot{V}o_2$ max, the latter determined on bicycle ergometer according to Åstrand and Saltin (1961), were 2 yrs, 179±1 cm, 69.9±1.8 kg, and 4.2±0.1 l min⁻¹ respectively

Muscle biopsies (Bergstrom 1962) for histochemical analysis were taken from the vastus lateralis m of the left leg at rest. Histochemical staining for myofibrillar ATPase was performed after preincubation pH 10.3 (Padykula and Herman 1955 Guth and Samaha 1969). Muscle fibres were classified in one the two main categories, *i.e.* as fast twitch (FT or type II) fibres or slow twitch (ST or type I) fibres (Er 1962). For classification into subgroups FT a and FT b (type II A and type II B), muscle fibre sect were preincubated at pH 4.6 and 4.3 according to Brooke and Kaiser (1970). Muscle fibre area w determined on muscle transverse sections stained for NADH-diaphorase activity (Novikoff *et al.* 19 based on a method described by Thorstensson (1976). The relative area of FT fibres was calcula according to the formula (100 FT/ST area FT) (FT/ST area FT + %ST)⁻¹

The subjects performed repeated maximal isokinetic knee extensions with the left leg on 2 differ occasions within a week. The angular velocity of their knee extensions corresponded to 3.14 rad (180 s⁻¹) with a frequency of approximately 50 contractions min⁻¹. This exercise was performed w the subject sitting in fixed position with his leg attached to the lever arm of isokinetic appara (Cybex II Lumex Inc., New York, USA). The test procedure and its accuracy and reliability have be described in detail elsewhere (Thorstensson 1976). The first session consisted of 50 muscle contraction The second session was terminated after 25 contractions. Peak torque in each contraction was record continuously

The highest torque value obtained during the first 5 contractions was defined as initial strength. T mean value for the last 3 contractions in the experiments was compared with initial strength, and bo absolute and relative decline in peak torque were calculated.

Muscle biopsies for biochemical analysis were taken from m. vastus lateralis within 3–4 s after 50 ar 25 knee extensions respectively. The biopsies were frozen in liquid nitrogen and stored at −80 C un analysed. Lactate concentration, total LDH and LDH isozyme activities were analysed on freeze dried ar dissected pools of the two main fibre types according to Tesch *et al.* (1978).

Means, standard error of the means (S.E.) and linear regression coefficient (r) were calculated. Differences between means were tested for significance using "Student's t" test.

Results

Initial strength averaged 161 Nm (range 117–237). Mean decline in peak torque after 25 contractions amounted to 40 Nm (range 20–75) or 25% (range 16–50) of the initial value. No significant differences between the two experimental occasions were observed for initia

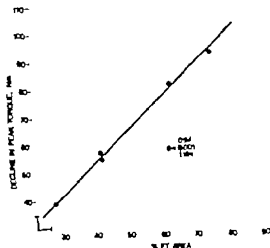


Fig. 1 The relationship between the relative distribution of FT muscle fibres in the vastus lateralis muscle and the decline in peak torque with 50 maximal knee extensions at an angular velocity of 3.14 rad s^{-1} (9).

...ngth or decline in peak torque after 25 contractions. The mean decrease obtained after ... contractions was 69 Nm (range 39–96) representing a 43% (range 29–59) decline.

Mean muscle fibre type distribution in the present group averaged 48% (range 29–76) FT fibres. FT a and FT b fibre distributions amounted to 29 (range 9–49) and 16% (range ...–39) respectively. Three per cent of the fibres counted were classified as unidentified. The relative cross-sectional area occupied by FT fibres averaged 53% (range 28–76).

Both initial strength and decline in peak torque after 50 contractions were positively correlated to the relative distribution of FT fibres, expressed either as the percentage of FT fibres (r 0.88, p 0.001) or as the relative area occupied by FT fibres (r = 0.94 p < 0.001 Fig. 1). No correlation was present between torque decline after 25 contractions and muscle fibre type distribution (Table I). It was not possible to demonstrate any differences in initial strength or decline in peak torque between subjects with a similar muscle fibre type distribution but differing in the FT a/FT b ratio. Thus, the present study did not confirm earlier findings obtained in animal experiments (Burke and Edgerton 1975).

LDH_{tot} activity averaged 1.11 (range 0.57 1.82) 10^{-4} and 0.61 (range 0.33–1.11) 10^{-4} mmoles mm^{-1} kg^{-1} wet weight in FT and ST fibres respectively. The corresponding values obtained for M-LDH were 0.86 (range 0.37 1.76) 10^{-4} and 0.30 (range 0.11–0.81) 10^{-4} mmoles min^{-1} kg^{-1} wet weight.

The muscle lactate concentration in FT fibres averaged 22.2 (range 4.0–31.0) mmoles kg^{-1} wet weight after 25 contractions and 29.0 (range 11.7 50.5) mmoles kg wet weight after 50 contractions. Values obtained in ST fibres were 13.7 (range 3.4–30.5) and 27.0 (range 10.4–53.8) mmoles kg^{-1} wet weight respectively.

A significant difference in lactate concentration was found between FT and ST fibre types after 25 contractions (p 0.01), but this lactate concentration gradient between the two fibre types equalized after 50 contractions (Fig. 2). The FT/ST lactate ratio, i.e. the lactate concentration in FT fibres over the lactate concentration in ST fibres was 1.44 (range 1.18–2.00) after 25 contractions and 1.14 (range 0.88–1.82) after 50 contractions.

relation to muscle fatigue (Karlsson 1971 Simonson 1971). Tesch *et al* (1978) recently demonstrated a relationship between muscle lactate accumulation and the percentage of FT fibres after 25 maximal voluntary contractions, using the same experimental set-up cited above, indicating a higher lactate production in FT fibres than in ST fibres. Higher activity of the lactate forming enzyme, lactate dehydrogenase (LDH) and a LDH isozyme pattern more in favour of lactate production have also been demonstrated in FT as compared to ST fibres (Sjödin 1976, Thorstensson *et al* 1977)

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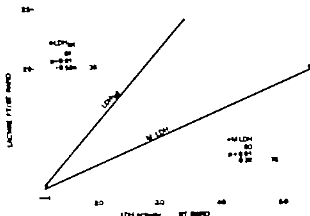


Fig. 1. The relationship between FT/ST lactate ratio, following 25 contractions, and FT/ST ratios for total LDH and M-LDH activities respectively

muscle fibres (Essén *et al.* 1975 b, Sjödin 1976). Higher activities of glycolytic enzymes, e.g. LDH and M-LDH (Sjödin 1976, Thorstensson *et al.* 1977), would favour a more rapid lactate formation in FT fibres (Tesch *et al.* 1978). Great differences in the LDH and M-LDH activity between the fibre types, as was found in the present study appear to create conditions for the formation of high lactate gradients between fibre types even if both FT and ST motor units are recruited at onset of exercise (*cf.* Desmedt and Godaux 1977). In addition, there may be a lower rate of lactate release from the FT fibres into the blood stream, since these fibres are normally surrounded by fewer capillaries than ST fibres (Andersen 1975).

The lactate concentration of the fibre types had equalized after 50 contractions (approximately 60 s). This may have been due either to a general inhibition of the glycolysis or to a specific inhibition of the myofibrillar enzyme system in the FT fibres (see below). This would in turn result in an increased reliance on ST fibres (which might increase the rate of glycolysis and lactate formation also in this fibre type). In addition, there may be a lactate flow from FT to ST fibres as has been discussed by Essén *et al.* (1975 a), possibly mediated by membrane-bound M LDH (Siesjo *et al.* 1968, Sjodin *et al.* 1976). The rate of lactate flow and equalization of the lactate gradient between the fibre types should then be related to M-LDH activity in both FT and ST muscle fibres.

A correlation between muscle lactate concentration and muscle fatigue during 15 min stimulation of frog muscle was recently demonstrated by Fitts and Holloszy (1976). However due to a discrepancy between muscle force and lactate concentration during recovery the authors excluded lactate itself as the direct cause of muscle fatigue. Instead associated pH changes were suggested as one possible factor

In human skeletal muscle biopsy homogenates good correlations have been demonstrated between lactate concentration and pH (Hermansen and Osnes 1972, Sahlin *et al.* 1976). After heavy exercise pH values in the order of 6.4–6.6 were reported corresponding to [concen]trations of 25–35 mmol kg^{-1} wet weight. In the present study mean value for lactate concentration in FT fibres after 25 contractions was 22.2 mmol kg^{-1} wet weight. It seems plausible that similar, or even higher lactate concentrations could be present in certain

TABLE I Correlation coefficients (r) and levels of significance (p) for some variables investigated. (n 9

	25 contractions		50 contractions	
	r	p	r	p
Force decline[1] versus:				
% FT area	0.59	n.s.	0.94	<0.001
lactat concentration	0.37	n.s.	0.60	n.s.
LDH_{tot} activity[a]	0.52	n.s.	0.82	<0.01
M LDH activity[3]	0.53	n.s.	0.84	<0.01
lactate concentration, FT/ST	0.71	<0.05	0.30	n.s.
LDH_{tot} activity FT/ST	0.91	<0.001	0.54	n.s.
M LDH activity FT/ST	0.76	<0.05	0.46	n.s.
Lactat concentration, FT/ST versus				
LDH_{tot} activity FT/ST	0.81	<0.01	0.01	n.s.
M LDH activity FT/ST	0.80	<0.01	0.38	n.s.

Nm. mmol kg⁻ wet muscle. *10^{-4} mmol min kg^{-1} wet weight.*

This difference was not statistically significant However in 8 of the 9 subjects the FT/S lactate ratio decreased The muscle lactate FT/ST ratio after 25 contractions was positivel correlated both to the corresponding *FT/ST ratios for LDH_{tot} activity and M LDH activi* (Fig. 3 Table I) A high lactate ratio was accordingly accompanied by a high LDH and M LDH ratio respectively (Fig. 3) Furthermore, these variables were both correlate to decline in peak torque after 25 contractions (Fig. 4 and 5). Similar relationships wer not found after 50 contractions (Table I)

Discussion

In conformity with earlier studies with the same experimental design (Thorstensson an Karlsson 1976, Nilsson *et al* 1977), significant correlations were found between the relativ incidence of FT muscle fibres, on the one hand, and initial strength and decline in peak torqu at a high contraction velocity on the other hand

The higher lactate concentration in FT fibres observed after the initial 25 rapid dynami contractions could be due to a primary (*Grimby and Hannerz 1968*) *recruitment of F* motor units (Burke and Edgerton 1975) and/or to a higher glycolytic capacity of F

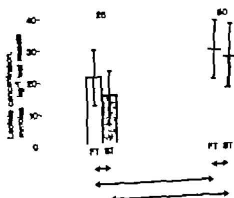


Fig. . Lactate concentration in FT (open bars) and ST muscle fibres (shaded bars) after 25 and 50 contractions, respectively. Values are means ± S.E. (n = 9). The number of stars denotes the level of significance (p < 0.05, p < 0.01 p < 0.001) of the differences indicated by the arrows.

lactate in FT fibres. Concomitantly observed differences in the activity of LDH and LDH isozymes between fibre types may influence both the formation of lactate (and H^+ ions) and the transport of lactate between fibre types, thus indirectly affecting contractile ability and fatigue.

This study was supported by grants from the Swedish Medical Research Council (B77-04X-4251-04A, [illegible]) and the Research Council of the Swedish Sports Federation.

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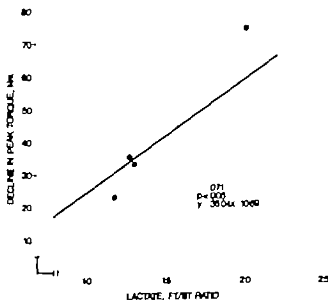


Fig. 4 The relationship between decline in peak torque and FT/ST lactate ratio following 25 contractions.

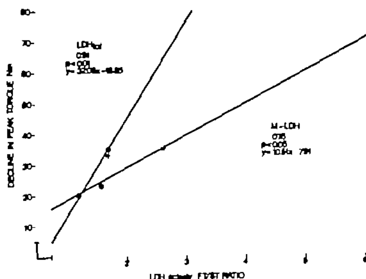


Fig. 5. The relationship between decline in peak torque following 25 contractions and FT/ST ratio for total LDH and M LDH activity respectively

extremely fatiguable FT fibres already after 5–10 contractions causing failure to produce tension and decline in force either directly or via a decrease in pH

A mechanism for the high H concentrations to cause impaired muscle function has been demonstrated by Fuchs *et al.* (1970) and Nakamura and Schwarz (1972). They showed that an increase in the H concentration decreases the number of calcium ions bound to troponin during excitation—contraction coupling. This would reduce the number of active actin-myosin interactions, thus decreasing contractile force. Moreover in histochemical studies (*e.g.* Brooke and Kaiser 1970, *cf* Methods) it has been observed that the myofibrillar ATPase activity of FT fibres is more sensitive to high H concentrations than that of ST fibres, which also may have functional implications.

To summarize, the present results indicate that initial impairment of muscular function during repeated fast contractions is accompanied by an initial intracellular accumulation of

lower in FT fibres. Concomitantly observed differences in the activity of LDH and LDH isozymes between fibre types may influence both the formation of lactate (and H^+ ions) and the transport of lactate between fibre types, thus indirectly affecting contractile ability and fatigue.

This study was supported by grants from the Swedish Medical Research Council (B77-04X-4251-04A, B78-04X-4251-05B) and the Research Council of the Swedish Sports Federation.

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Transient water diuresis and syndrome of inappropriate antidiuretic hormone secretion (SIADH) induced by forebrain lesions of different location

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Received 20 January 1978

Abstract

RUNDGREN, M. and F. FYHRQUIST. *Transient water diuresis and syndrome of inappropriate antidiuretic hormone secretion (SIADH) induced by forebrain lesions of different location.* Acta physiol. scand. 1978. *103* 421–429

Inhibition of ADH-secretion and transient water diuresis was observed as acute effects of radio-frequency lesions in the septal region of goats. The water diuresis was not compensated for by drinking and therefore rapidly induced pronounced hypernatremia and hypovolemia. The development of hypovolemia was accompanied by a rise in plasma renin activity. Lesions of the same kind, but extending into the preoptic region near the medial portion of the supraoptic nuclei induced the inability to excrete excessive water characteristic of SIADH. Determinations of plasma arginine vasopressin suggested that the lesions causing SIADH did not produce any noticeable increase in basal ADH-secretion. The results suggest that impulses from periventricular receptors regulating ADH-release and water intake to a considerable extent are transmitted via the septal region, and that elimination of this impulse traffic is sufficient to turn water balance to the negative side. However, reflex volumetric inhibition of the ADH-secretion does not seem to be mediated by pathways passing through the septal region.

A sensitive regulation of antidiuretic hormone (ADH) secretion is of essential importance for the maintenance of normal body fluid composition and volume. According to the present views (*cf* Andersson 1977), the main features of this regulation are as follows. The principal factors which determine the rate at which ADH is released from the neurohypophysis are the [Na^+] of the extracellular fluid and the blood volume. ADH-release in response to normal or elevated extracellular [Na^+] is apparently mediated by sodium sensitive receptors which predominantly are located near the anterior wall of the third cerebral ventricle. The volume regulation by all probability consists of both neural and humoral links. Impulses from cardio-vascular distention receptors and the baroreceptors seem to exert a tonic inhibition of ADH-secretion, whereas hypovolemia-induced renal renin release, with subsequent formation of angiotensin II, stimulates ADH-secretion. This effect of angiotensin II appears to be mediated by the same cerebral receptors which are affected by the extracellular [Na^+].

Disturbances in this well balanced regulatory system may lead to an uncontrolled ho[illegible] mone release so-called inappropriate ADH-secretion. The syndrome of inappropria[illegible] ADH secretion (SIADH) was first described by Schwartz *et al* (1957) in man. Man is al[illegible] the only mammalian species in which SIADH is likely to become clinically manifest, sin[illegible] substantial fluid intake, not motivated by thirst, as a rule is the prerequisite for the develo[illegible] ment of the syndrome. The main characteristics of SIADH are hyponatremia, plasm[illegible] hypo-osmolality and hypervolemia, together with inability to eliminate with the urine [illegible] excessive load of water (*cf* Bartter and Schwartz 1967) This syndrome has now and the[illegible] been observed in connection with CNS disorders in humans (*cf* Bartter 1973) and has al[illegible] been described as an effect of experimentally induced forebrain lesions in the goat (Andersson, Leksell and Lishajko 1975) Another "release phenomenon" concerning water balanc[illegible] observed in that study was the septal polydipsia originally discovered in the rat by Harv[illegible] and Hunt (1965)

The intention of the present study was to make a more thorough investigation of SIAD[illegible] induced by forebrain lesions, and to study whether lesions mainly restricted to the sept[illegible] region might influence the secretion of ADH

Methods

Animals. 7 female goats (pre-lesion b wt 30–40 kg) were used. The animals were kept in metabolism cages (separating urine from feces) [illegible]t room temperat[illegible]re (20 ± 1°C), where they had free access to hay and water In order to maintain the goats in positive sodium balance, they were given 6 g of NaCl added to a dail[illegible] ration of 400 g of commercial grain mix. The [illegible]4 h w[illegible]ter intak[illegible] and urine output were measured round 8 a.m. when samples of the urine were taken for analyses.

Electrode implantatio[illegible] 5 of the goats had a pai[illegible] of thermo-couple electrodes (o.d 0.7 mm) permanently implanted at various levels into the forebrain. The implantations were made under general (nembutal) anaesthesia 2 5 weeks before the electrodes were used for production of radio-frequency (RF) lesions (*cf* Galo 1963). All five animals had the two electrodes bilaterally impla[illegible]ted with an interspace of 3 or 4 mm The length of the uninsulated ends of the [illegible]lectrodes varied between animals from 3 t[illegible] 5 mm.

Production [illegible]f forebra[illegible] lesions. Coagulative lesions were made by [illegible]pplying RF energy between the un-insulated ends of the thermo-couple electrodes, having their temperature recordi[illegible]g units [illegible]onnected to an Ellab thermometer The tissue temperat[illegible]re between the electrode ends was elevated to 65°C for 5 min The RF heating was not seen to cause any pain or distress to the animals. Therefore, lesioning was made in the unanaesthetized goat standing in its habitual environment.

Hydration and [illegible]ater supplementation. Hydration wa[illegible] accomplished by giving 75–150 ml/kg of 38°C water by stomach tube into the rumen. Water supplementation was regularly administered in the same manner to one of the goats which developed permanent adipsia as an effect of the forebra[illegible]n damage.

Routine sampling and analyses During hydration experiments, and on the day of lesioning, urine was collected via a retention catheter inserted into the urinary bladder Jugular [illegible]ein blood samples (10 ml) were drawn into heparinized syringes. For determination of the hematocrit value (Ht) the samples were centrifuged in graded tubes at 3 000 rpm for exactly 7 min. Urine and plasma [Na[illegible]] [illegible]nd [K[illegible]] were measured by use of an IL 343" flame photometer and an Advanced Instruments In[illegible] osmometer was used for determination of the osmolality of these fluids.

Hormone analyses. Ice-chilled syringes with 0.3 *M EDTA* as anticoagulant [illegible]ere used t[illegible] obtain blood samples for radioimmunoassays. Determinations of plasma [illegible]rginine vasopressin (AVP) were performed with a method previously applied for determination of this hormone in huma[illegible] plasma (Fyhrquist Wallenius and Holkemans 1976 b). Cross-reaction with oxytocin (Sandoz) was < 0.03 %, with pressinoic acid (Ferring) < 0.01 %, and with C-terminal tripeptide of vasopressin (Ferring) < 0.01 %. Sequential saturation and the use of the Sigma VII AVP for standards and preparation of ^{125}I labelled tracer increased the assay sensitivity to 0.2 pg/ml. Recovery of synthetic AVP added to pooled goat plasma was 67–98 [illegible] in the low range (2.8 pg/ml added), and 82 105 % in the high range (28 pg/ml added).

Radioimmunoassay of plasma renin activity (PRA) were performed with method modified for goat plasma (Eriksson et al. 1976).

Administration of hormones. In order to imitate SIADH, prolonged (50–100 min) i infusions of synthetic AVP (Vasopressin Grade V Sigma, 80–340 pg/kg min) were made in association with hydration in two goats not used for ablation studies. Hydrocortisone ("Solu-Cortef" Upjohn), was given intravenously to one of the goats which had developed SIADH.

Histology. At the termination of the post-lesioning observation period the goats were decapitated under arterial anesthesia and the heads were perfused with isotonic saline followed by 8% formol saline. After fixation, block of the brain (including the preoptic region, the septum, the thalamus and the hypothalamus) was embedded in celloidine and cut by serial transverse (4 animals), or sagittal (one animal) sections at 30 µm. The sections were stained either with toluidine blue or according to Loyez (cf. Culling 67).

Figures followed by ± represent mean ± S.E.

Results

I. Pre-lesioning studies

During the periods between electrode implantation and lesioning (2 5 weeks) the daily water intake varied between animals from 25-50 ml/kg, but remained rather stable in each single goat. Urinary water loss was 50–75% of the intake and the range of urine osmolality was 600-1 600 mosm/kg. The renal Na and K excretion varied between 20–130 and 40–200 mmol/day respectively. Two or three blood samples taken in each goat during the pre-lesioning balance study revealed that the blood plasma composition remained rather stable. The following values (n = 13) were obtained [Na] 151 4 ± 0 6 mmol/l [K] 4.2 ± 0 1 mmol/l; osmolality 293 ± 2 mosm/kg. The Ht remained rather constant (range ± 5) in the single animal, but large interindividual differences were observed (28–39) Unfortunately pre-lesioning plasma AVP and PRA were only determined once in two of the goats (see tables)

The effect of hydration (100 ml/kg) was studied once or twice in each animal before lesioning (n 8). All goats developed positive renal free water clearance (C_{H_2O}) within one h and maximum water diuresis (C_{H_2O} = +5.8 ± 0.6 ml/min) 1.5 to 2 h after water loading. Blood plasma [Na] was then 148 ± 1 mmol/l and osmolality 290 ± 1 mosm/kg. Some increase in renal Na excretion (< 100%) was generally seen during the water diuresis.

II. Effects of forebrain lesioning

A. Transient water diuresis

Within the first post-lesioning hour two of the goats developed positive renal C_{H_2O}. The water diuresis reached maximum level 1 to 2 h later and lasted for 9 and 22 h respectively. Since the diuresis was not compensated for by drinking, the goats lost 3 1 and 3 6 liters of water and became markedly dehydrated as indicated by hypernatremia (160 and 163 mmol/l), plasma hyperosmolality (325 and 334 mosm/kg) and a 15% increase in Ht. In one of the goats plasma AVP and PRA were determined before, and at 4 and 10 h after lesioning. As shown in Fig. 1 the plasma AVP dropped from 11 to 3 pg/ml as an acute effect of the forebrain damage. The PRA rose from 1 1 to 2.7 ng/ml.h⁻¹ between the 4th and 10th post lesioning h when the goat had become markedly dehydrated and hypovolemic.



Fig. 1 Inhibition of the ADH-secretion (drop in plasma arginine vasopressin, PAVP) and transient water diuresis (positive renal C_{H_2O}) seen as acute effects of radio-frequency lesioning (*L*) in the septal region. Since the water diuresis is not compensated for by drinking it induces hypernatremia and hypovolemia, as indicated by the figures for plasma [Na] (in mmol/l) and hematocrit (Ht) on top of the figure. That the developing hypovolemia stimulates the renin-angiotensin system is evidenced by the rise in plasma renin activity (PRA) toward the end of the water diuresis.

One of the goats did not compensate for its water loss by spontaneous drinking until 4 days post lesioning which necessitated temporary water supplementation. It remained hypodipsic during the entire post lesioning observation period (50 days) with constantly elevated plasma [Na] (158 ± 1 mmol/l) (n = 10) When repeatedly subjected to water loading during this period the goat developed positive renal C_{H_2O} at a plasma [Na] of 155 mmol/l and plasma osmolality of 305 mosm/kg. The other goat started to drink again on the day after lesioning but its plasma [Na] and osmolality did not return to normal values until 10 days later At that stage the animal responded to water loading with a water diuresis in an apparently normal manner

Forebrain lesions The cerebral ablation in common for the goats which developed temporary water diuresis is demonstrated on a midsagittal section and three transverse sections in Fig. 2 (*vertically hatched* area) It involved the medial part of the septum and extended just in front of the anterior commissure to a horizontal level passing through that structure.

B *Apparent SIADH*

Three of the lesioned animals did not display any acute change in renal water excretion, but developed symptoms characteristic of inappropriate ADH secretion During the post lesioning observation periods (16, 20 and 90 days) the animals were repeatedly (n = 11) subjected to water loading (75 to 150 ml/kg) Although in normal water balance when the water load was administered, none of the goats responded with a water diuresis. Instead, hydration

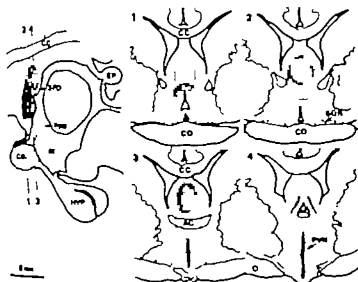


Fig. 2. A midsagittal section through the hypothalamic region of the goat (left), and four transverse sections (corresponding to the numbered levels on the sagittal section). The *vertically hatched* areas represent the cerebral damage in common for two goats in which RF-lesioning induced transient, uncompensated water diuresis. The *horizontally hatched* areas show the forebrain damage in common for three goats which developed SIADH after lesioning. III – Third cerebral ventricle; AC – Anterior commissure; CC – Corpus callosum; CM – Corpus mamillare; CO – Chiasma opticum; EP – Epiphysis; HYP – Anterior lobe of the hypophysis; OT – Optic tract; PVN – Paraventricular nucleus; S – Septal region; SFO – Subfornical organ; SON – Supraoptic nucleus.

induced pronounced hyponatremia, plasma hypo-osmolality, hypervolemia (as indicated by reduced Ht), and natriuresis. These effects of hydration are illustrated in Fig. 3.

The glomerular filtration rate (GFR) was not determined. Since reduced GFR, due to deficiency of glucocorticoids, could possibly have contributed to the animals' inability to

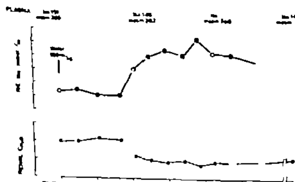


Fig. 3. Apparent inappropriate ADH-secretion (= inability to eliminate with the urine an excessive load of water) in a goat 18 days after a preoptic lesion of the extent shown in Fig. 2 (horizontal hatching) was made. Positive renal C_{H_2O} does not develop (lower curve) in spite of a progressive hemodilution (as indicated by the values for plasma [Na^+] in mmol/l and osmolality in mosm/kg shown on top of the figure). Note the pronounced increase in renal Na excretion (upper curve) which seems to be an effect of the abnormal expansion of the body fluid volume. Water given by stomach tube into the rumen at zero time

TABLE I

	Pre-lesion	Post-lesion		
	Non-hydrated	Non-hydrated	Hydrated	
	pl. Na 150.0 mmol/l (n = 1)	pl. Na 150.0 ± 0.8 (n = 8)	pl. Na 140–145 (n = 11)	pl. Na 14 (n = 6)
PAVP (pg/ml)	3.10	3.90 ± 0.68	3.07 ± 0.42	2.92 ± 0.48
PRA (ng/ml.h⁻¹)	1.19	1.27 ± 0.29	0.43 ± 0.06	0.30 ± 0.04

Plasma arginine vasopressin (PAVP) values, and values for plasma renin activity (PRA) obtained in one the goats which developed the syndrome of inappropriate ADH secretion (SIADH). The slight reduction AVP seen during post lesion hydration is not statistically significant, whereas the corresponding reducti in PRA is clearly significant ($p < 0.01$). pl. Na = blood plasma [Na].

excrete excessive water 125 mg of hydrocortisone was given intravenously to one of t goats 2 h after the administration of water In spite of this treatment the renal C_{H_2O} remain negative. However it seems more likely that the GFR, if anything, increased concomita with the hydration-induced expansion of the blood volume. It might explain why the ren Na excretion reached 3 to 6 times pre-hydration level within 3 h after water loading (Fig.

The reason why the post-lesioning observation periods were relatively short in two the goats (16 and 20 days) is that in final hydration experiments both animals were giv 150 ml/kg of water Since even this conspicuous hydration did not induce a water diures the animals developed symptoms of water intoxication (mild convulsions and signs pulmonary edema) For ethical reasons the animals were sacrificed as soon as these sym toms became apparent. Half an hour earlier the plasma [Na] had fallen to 120 and 1 mmol/l and the plasma osmolality to 260 and 264 mosm/kg.

AVP and PRA determinations Plasma AVP and PRA were only determined in the go studied for 90 days after lesioning. Unfortunately only one determination of the hormone was made during the control period. On that occasion the animal was non-hydrated Hence the hormonal response to hydration was not studied under normal conditions. However several determinations of plasma AVP and PRA were made after the brain damage ha been induced, both in the non-hydrated and hydrated animal. The results shown in Table indicate that the forebrain damage did not cause any notable rise in non-hydration AVP o PRA values. After lesioning, hydration seemed to induce a slight fall in plasma AVP bu it was statistically insignificant. In contrast, the concomitant fall in PRA was clearly signi ficant ($p < 0.01$)

Thirst defects. The goat in which plasma AVP and PRA were determined became adipsi as effect of its forebrain damage and had to be maintained on daily water supplementation All experiments made to investigate the animal's response to an excessive load of water were made on occasions when previous water supplementation had restored pre-lesioning water balance (= normal b wt., plasma [Na], osmolality and Ht). The same precautions were undertaken before hydrating one of the other goats which had become hypodipsic after lesioning.



Fig. 4. A transverse section (30 μ) at the level of the anterior commissure (a.c.) showing the bilateral and ventral extent of the forebrain lesion in one of the goats which developed SIADH. Note that the ventral border of the lesion on both sides is not far from the medial portions of the supraoptic nuclei (SON). The extent of these nuclei is indicated by broken lines. The parts of the anterior commissure (a.c.) which are not directly affected by the lesion have degenerated. CC = Corpus callosum; CO = Chiasma opticum. [illegible]

Forebrain lesions. The main difference in location between the lesions causing temporary water diuresis (see above) and those resulting in SIADH was that the latter extended down into the preoptic region. The brain damage in common for the three "SIADH" goats is shown in Fig. 2 (*horizontally hatched* areas). As seen in transverse section no 2 of this figure, and in Fig. 4, the ventral border of the lesions was not far from the medial portion of the supraoptic nucleus on both sides of the brain. In the adipsic goat the cerebral damage extended in posterior direction to involve almost the entire frontal wall of the third ventricle. Only the upper half of the frontal ventricular wall was affected by the lesion in the hypodipsic animal.

III. *Imitation of SIADH in control animals*

Prolonged (50 to 100 min) infusions of synthetic AVP were made in connection with hydration on four occasions in two control goats. The effect on fluid balance was very similar to that observed when the "SIADH" goats were hydrated (*cf.* Fig. 3). Thus, when the AVP infusion was started at the same time as water (100 ml/kg) was administered (n = 2), positive renal C_{H_2O} did not develop until 90 min after discontinuation of the infusion. In the meantime, renal Na^+ excretion rose to about 5 times pre-infusion level and considerable hyponatremia (140 mmol/l) and plasma hypo-osmolality (270 mosm/kg) developed. Likewise, when the AVP infusion was started 1 h after water loading (n = 2), the resulting, long-lasting inhibition of the water diuresis was accompanied by pronounced natriuresis and hemodilution.

Discussion

A definite proof that the release of ADH from the neurohypophysis is regulated by ne impulses from the hypothalamus was provided already forty years ago by Fisher Ingr and Ranson (1938), who demonstrated that transection of the median eminence cau diabetes insipidus in the cat. Since then, experimental diabetes insipidus has been indu in the same manner in many mammalian species, and it has been shown that the posit renal C_{H_2O}, characteristic of diabetes insipidus, might develop within ? h after RF-coagu tion of the median eminence (Olsson 1970) In all these instances, the blockage of the AD release in non-hydrated animals has been due to interruption of the final neuronal link the ADH-release mechanism (the axons of neurons in the supraoptic and paraventricu nuclei) However a temporary water diuresis, uncompensated by drinking, was recen observed in two goats as effect of forebrain RF-lesions which did not involve the neur of the supraoptic and paraventricular nuclei (Andersson *et al* 1975) In these animals t medially placed lesions, which destroyed most of the anterior wall of the third ventric also caused permanent adipsia. It was suggested that the brain damage had inactivated cerebral sensory mechanism regulating water intake and ADH-secretion, but had left t reflex volume regulation of the ADH-release in gear Unexpectedly a similar acute po lesioning water diuresis was observed here as effect of damage mainly confined to the sep region Determinations of plasma AVP made in one of the animals (Fig. 1) revealed that t water diuresis was in fact due to a retardation of the basic ADH release occurring in no hydrated, but water replete animals. The brain damage in these goats obviously also i paired a regulation of water intake dependent upon extracellular [Na] and circulati angiotensin II Thus, the excessive acute water loss was not compensated for by drinking spite of a progressive rise in plasma [Na] and hypovolemia induced activation of t renin-angiotensin system (indicated by the increase in PRA shown in Fig. 1). Since th cerebral damage in these animals did not include the anterior wall of the third ventricle, appears unlikely that juxtaventricular receptors involved in the control of water balanc (*cf* Andersson 1977) were destroyed to any appreciable extent. More likely is that the sept lesions interrupted pathways transmitting stimulatory impulses from juxtaventricula receptors to the supraoptico-neurohypophyseal system. Only afferents from recepto located near the anterio-dorsal wall of the third ventricle or further posterior along th cerebroventricular system seem to have been involved since the forebrain damage did no extend below the level of the anterior commissure (Fig. 2). However receptors regulatin ADH-release and water intake are apparently present also near the ventral portion of th third ventricle, *i.e.* below the level of the anterior commissure (*cf* Andersson 1977) There fore, the effects of septal lesions observed in the present study indicate that elimination o afferent impulses from only a certain proportion of the juxtaventricular receptor population is sufficient to turn water balance to the negative side.

20 years ago Walker (1957) suggested that the apparent inappropriate secretion of ADH seen in hydrated subjects during anaesthesia is due to removal of a tonic inhibition of spon- taneous intrinsic activity in the supraoptic neurohypophyseal system. That autonomous activity may be present in this final neuronal link of the ADH-release mechanism has later been indicated by electrophysiological recording of discharges in supraoptic nuclei isolated

[illegible] the rest of the brain (Suda, Koizumi and Brooks 1963). It appears likely that cardio-vascular detection and pressor receptors (cf Gauer Henry and Behn 1970) may exert a [illegible] inhibition of the ADH-secretion in the manner originally suggested by Walker (1957). [illegible] of this kind of tonic inhibition was suggested to have caused the apparent in-[illegible] ADH-release previously observed as effect of forebrain lesions encroaching upon the supraoptic nuclei in the goat (Andersson *et al.* 1975). It also seems to be a plausible explanation of the SIADH observed in the present study which provides some additional information on the matter The fact that extensive septal lesions as such, did not impair the ability to eliminate excessive water implies that reflex volumetric inhibition of the ADH release predominantly is mediated by pathways not passing above the anterior commissure.

The plasma AVP determinations made in one of the "SIADH" goats do not allow any far-reaching conclusions, since only one AVP value was obtained before lesioning. These few data are in accordance with the previous observation in man (Miller and Moses 1972) that SIADH may be present without any noticeable increase in the basic ADH-secretion. Further studies are, however obviously needed to disclose whether this is characteristic of SIADH induced by forebrain lesions.

This work was supported by grants from Karolinska Institutet, from Svenska Sällskapet för Medicinsk Forskning, and from the Finnish National Research Council for Medical Science (Project No 2228/3).

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Acta physiol. scand. 1978. 103 430–436

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The distensibility of the resistance vessels of skeletal muscle in hypertensive patients

By

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Received 24 January 1978

Abstract

HARTLING O T L. SVENDSEN P E. NIELSEN and J TRAP-JENSEN. *The distensibility of resistance vessels of skeletal muscle in hypertensive patients* Acta physiol. scand. 19 *103* 430–436

The distensibility of the resistance vessels of skeletal muscle was evaluated in 23 patients with essen arterial hypertension (WHO grade I II), and I 14 normotensive control subjects. Five of the hypertens patients were well treated for over 2 years and 18 were untreated. The 133Xenon wash-out rate from t a terior tibial muscle during reactive hyperemia was recorded before and during an increase of the vasc transmural pressure brought about by application of a subatmospheric pressure to the leg. At ambie pressure the 133Xenon wash-out rat did not differ between the normotensive and hypertensive subjec reflecting equal muscle blood flows. However when the transmural pressure was augmented the 133Xen wash-out rate in the ormotensive subjects increased about twice as much as in the hypertensive patien This suggests a decreased distensibility of the resistance vessels in hypertensive patients as compared normotensive subjects, and supports the concept that structural changes of these vessels tak place arterial hypertension. Since the vascula distensibil ty was equally reduced in the untreated and treated hypertensive patients it is indicated that the structural changes in the resistance essels of the k muscles do not readily decline during antihypertensiv treatment.

Key words: Arterial hypertension, muscle blood flow reactive hyperemia, vascular compliance vascula transmural pressure 133Xenon wash-out technique

Chronic arterial hypertension seems to be associated with a reduced vascular distensibility In animals with arterial hypertension *in vivo* and *in vitro* studies have demonstrated a decreased distensibility (or compliance) of the aorta, the femoral artery and the resistance vessels (Feigl *et al* 1963 Aars 1968 Hallbäck *et al* 1974) Although the decreased distensi bility apparently applies to the entire arterial system the changes of the resistance vessels are undoubtedly of primary importance from a hemodynamic point of view as the major pressure drop from the aorta to the central veins occurs here.

In hypertensive human subjects *in vitro* studies have shown a decreased distensibility (or increased stiffness) of the aorta and the carotid sinus (Hallock and Benson 1937

Ameroth and Kreuziger 1951). Moreover a study of the exposed brachial artery has demonstrated a smaller *in vivo* distensibility of this artery in hypertensive than in normotensive subjects (Greene *et al.* 1966).

An investigation of the distensibility of the resistance vessels in arterial hypertension has so far not been undertaken in man. A main difficulty of evaluating the true distensibility of the resistance vessels *in vivo* is avoiding reflex constriction of vascular smooth muscles when the distending pressure is augmented. However during reactive hyperemia after severe ischemic exercise, the resistance vessels behave like passive-elastic tubes to changes in the vascular transmural pressure, suggesting that all vasoconstrictor activity is abolished (Harting *et al.* 1976, Neubauer 1977). Thus, in this situation it seems possible to evaluate the inherent elastic properties of these vessels.

In the present study the distensibility of the resistance vessels in the anterior tibial muscle was evaluated in untreated and well treated hypertensive patients, as well as in normotensive subjects. This was accomplished by recording the increase in regional muscle blood flow caused by an increase in the vascular transmural pressure during reactive hyperemia.

Material and methods

The study comprised 18 patients (12 males and 6 females) with essential hypertension (WHO grade I–II (WHO 1978)) receiving no antihypertensive treatment, 5 patients (4 males and 1 female) in the same state of disease but carefully treated with adrenergic beta-receptor blocking agents and diuretics for more than two years, and 14 healthy normotensive control subjects (11 males and 3 females). The age, height, and weight distributions were approximately the same in the three groups. (Mean age 41 years; range 27–60 years.)

All of the subjects consented to participate in the study after being informed in detail of its nature and purpose.

The patients were all from the out-patient clinic. The clinical examinations included and X-ray study of the heart, ECG, ophthalmoscopic examination of the eye fundus, test for proteinuria, urine macroscopy, [illegible] wash-out urography or radioisotope renography and determination of plasma creatinine and electrolytes.

In each subject peripheral pulses were palpated (a. dors. ped., and a. tib. post.), and the systolic blood pressure at the level of the ankle and the first toe were measured by mercury-in-silastic strain gauge plethysmography in order to ensure that no hemodynamic significant obliterative arterial disease of the lower extremities was present. In the supine body position the blood pressure at ankle level should not be lower than the systolic blood pressure at heart level measured sphygmomanometrically on the upper arm and the blood pressure of the first toe should not be lower than 30 mmHg below the systolic blood pressure (Nielsen *et al.* 1972).

The post ischemic blood flow of the anterior tibial muscle was evaluated by means of the ^{133}Xenon wash-out technique (Lassen *et al.* 1964, Harting *et al.* 1976). The subject was supine with the lower leg supported by a cushion so that the tibial muscle was at heart level. Approximately 0.2 ml of a sterile saline solution containing ^{133}Xenon (0.5–1 mCi/ml) was injected into the anterior tibial muscle. Ischemia of the lower leg was induced by means of an inflatable cuff (8.5 cm wide) placed around the thigh just above the knee and inflated to suprasystolic pressure (Fig. 1). After 3 min the subject performed ischemic exercise (rhythmic dorsi-plantar flexions of the unloaded foot) until severe pain made further movements impossible. Then the lower leg was enclosed in a rigid PVC tank that fitted airtight just above the knee without compression of the skin (Fig. 1). After deflation of the cuff the ^{133}Xenon wash-out curve was followed at ambient pressure for about 1 min. Then the pressure in the tank was lowered to 30 mmHg below atmospheric within 1–2 s and the effect on the wash-out curve observed for about 1 min (Fig. 1). For each subject the mean of the values obtained from the two legs was calculated.

During the period of reactive hyperemia the ^{133}Xenon wash-out curve was continuously recorded by a scintillation detector mounted about 20 cm from the leg and pointing at the area of the isotope deposit (Fig. 1). The detector was coupled to a channel analyzer, a logarithmic recording potentiometer and to a

Acta physiol. scand. 1978. 103. 430–436

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The distensibility of the resistance vessels of skeletal muscle in hypertensive patients

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Received 24 January 1978

Abstract

HARTLING O T L. SVENDSEN, P E. NIELSEN and J TRAP-JENSEN. *The distensibility of the resistance vessels of skeletal muscle in hypertensive patients* Acta physiol scand. 1978, *103* 430–436

The distensibility of the resistance vessels of skeletal muscle was evaluated in 23 patients with essential arterial hypertension (WSHO grade I II), and 14 normotensive control subjects. Five of the hypertensive patients were well treated for over 2 years and 18 were untreated. The 133Xenon wash-out rate from the anterior tibial muscle during reactive hyperemia was recorded before and during an increase of the vascular transmural pressure, brought about by application of a subatmospheric pressure to the leg. At ambient pressure the 133Xenon wash-out rate did not differ between the normotensive and hypertensive subjects reflecting equal muscle blood flows. However when the transmural pressure was augmented the 133Xenon wash-out rate in the normotensive subjects increased about twice as much as in the hypertensive patients. This suggests a decreased distensibility of the resistance vessels in hypertensive patients as compared to normotensive subjects, and supports the concept that structural changes of these vessels take place in arterial hypertension. Since the vascular distensibility was equally reduced in the untreated and well treated hypertensive patients it is indicated that the structural changes in the resistance vessels of the leg muscles do not readily decline during antihypertensive treatment.

Key words: Arterial hypertension, muscle blood flow reactive hyperemia, vascular compliance vascular transmural pressure 133Xenon wash-out technique

Chronic arterial hypertension seems to be associated with a reduced vascular distensibility. In animals with arterial hypertension *in vivo* and *in vitro* studies have demonstrated a decreased distensibility (or compliance) of the aorta, the femoral artery and the resistance vessels (Feigl *et al* 1963 Aars 1968, Hallbäck *et al* 1974) Although the decreased distensibility apparently applies to the entire arterial system the changes of the resistance vessels are undoubtedly of primary importance from a hemodynamic point of view as the major pressure drop from the aorta to the central veins occurs here.

In hypertensive human subjects *in vitro* studies have shown a decreased distensibility (or increased stiffness) of the aorta and the carotid sinus (Hallock and Benson 1937

Table I. Arterial mean blood pressure, ^{133}Xenon wash-out rate (k-value) from the anterior tibial muscle, and calculated "vascular resistance" (mean blood pressure/k-value) during reactive hyperemia in normotensive, and hypertensive subjects. Changes induced by exposing the leg to subatmospheric pressure of 50 mmHg. Mean values ± one standard deviation (S.D.) are presented.

		Mean blood pressure (mmHg)	^{133}Xenon wash-out rate k-value (min^{-1})	"Vascular resistance" (mean blood pressure/k-value) (mmHg·min)
Normotensive subjects (n 14)	mean	106 ± 11.1	0.90 ± 0.27	120.4 ± 29.3
	change	−1 ± 0.8	+ 0.34 ± 0.12	−38.0 ± 19.4
	per cent change		+41.2 ± 18.1	−28.3 ± 8.9
Hypertensive subjects (untreated) (n=10)	mean	136 ± 16.7	0.95 ± 0.18	146.3 ± 31.9
	change	−2 ± 4.1	+0.16 ± 0.11	−22.0* ± 13.6
	per cent change		+17.3 ± 13.2	−15.4 ± 9.4
Hypertensive subjects (treated) (n=9)	mean	109 ± 8.7	0.90 ± 0.30	129.1 ± 38.7
	change	−2 ± 2.4	0.14 ± 0.09	21.0* ± 19.6
	per cent change		+16.7* ± 14.9	−14.1 ± 10.0

* Significant difference between normotensive and hypertensive subjects, $P < 0.05$.
† Significant difference between normotensive and hypertensive subjects, $P < 0.01$

Discussion

The study demonstrates that the ^{133}Xenon wash-out rates (k-values) from the anterior tibial muscle during reactive hyperemia at ambient pressure are essentially the same in normotensive and hypertensive subjects. This indicates equal blood flows in normotensive and hypertensive subjects, but higher vascular resistance in the untreated hypertensive patients in proportion to the elevated blood pressure. These results are in good agreement with those obtained by Amery *et al.* (1969) using the same technique.

The main result of the present study is, however that patients with hypertension respond to a given increase in vascular transmural pressure (distending pressure) with a smaller increase in the ^{133}Xenon wash-out rate (Δk)—absolute and fractional—than do normal subjects. This reflects a smaller increase in the muscle blood flow. Since the variation in blood flow predominantly depends on the change in luminal diameter of the resistance vessels the finding of a smaller flow increase in response to increased transmural pressure suggests a reduced distensibility or compliance of these vessels in hypertension.

Evidently it is presupposed that the experimental situation (local anoxia created by the combined effects of arrested blood flow and muscular exercise), caused complete relaxation of the smooth muscle cells in the resistance vessels. Thus it is assumed that arteriolar constriction—which would normally be elicited by an increase in the vascular transmural pressure (*cf.* Henriksen, 1977)—was abolished by unresponsiveness of the vascular smooth muscles. Previous studies indicate that complete vascular relaxation can indeed be attained during reactive hyperemia both in normal and hypertensive subjects (Folkow *et al.* 1958, Conway 1963, Sivertsson 1970). Thus it has been shown that prolongation of the ischemic period or intraarterial infusion of the potent vasodilating substance, adenosinetriphosphate, do not further augment the muscle blood flow (Folkow *et al.* 1958, Conway 1963). Moreover

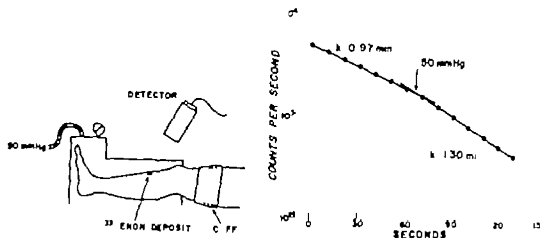


Fig. 1 *Left part* Diagram of the experimental setup. *Right part* Typical [133]Xenon wash-out curve from the anterior tibial muscle during reactive hyperemia in a normotensive subject before and during application of a subatmospheric pressure to the leg.

digital printer The half-time value t½ was read from the wash-out curve, and the [133]Xenon wash-out rate constant, the k-value in min⁻¹ calculated as $k = \ln 2/t½$.

Statistical evaluation of the data was made by means of the Student's t-test for paired and unpaired observations, and by means of correlation coefficient analysis. Differences were considered to be significant if a 2 P-value below 0.05 was obtained.

Results

At ambient pressure the [133]Xenon wash-out rates (k values in min⁻¹), during reactive hyperemia of the treated and untreated hypertensive subjects did not differ significantly from those of the normotensive subjects (Table I) As a corollary of the elevated blood pressure, estimated vascular resistance was higher in the untreated hypertensive subjects. In the small group of well treated hypertensive patients vascular resistance was lower than that of the untreated patients, but the difference was not statistically significant (Table I).

The [133]Xenon wash-out rate increased significantly ($p < 0.01$) both in normal subjects and in hypertensive subjects when the vascular transmural pressure was augmented by application of a subatmospheric pressure of −50 mmHg to the leg (Table I, Fig. 2). However this increase in the k value (Δk) was much greater in the normotensive subjects than in both untreated and well treated hypertensive patients, there being no difference of the Δk between the two hypertensive groups (Table I) Correspondingly the fall in estimated vascular resistance was far more pronounced in the normal subjects. There was a small but significant negative correlation between the arterial mean blood pressure and the increase in the [133]Xenon wash-out rate when the untreated patients and the normal subjects were taken together ($r = 0.55$ estimating equation $y = -0.004x + 0.71$ with y signifying Δk (min⁻¹) and X arterial mean blood pressure (mmHg).

Table I indicates that the arterial blood pressure in both normotensive and hypertensive subjects remained largely constant during the [133]Xenon wash-out measurements. However the blood pressures measured during the examination were 5–6% above the pre-examination levels in all groups, no doubt due to the unfamiliar experimental situation and the ischemic pain.

in the maintenance of the hypertensive state (cf Folkow et al 1973). Therefore it is an important question whether these changes are reversible or not. Studies of hypertensive rats have clearly shown that the vascular changes can be largely prevented by antihypertensive treatment provided this is instituted at an early age. If on the other hand, the animal is not treated until it is middle-aged the changes are only partially reversible (Weiss 1974). In arterial hypertension in man vascular structural changes of the hand, indicated by lowered blood flow resistance at maximal dilatation, were reported by Sivertsson (1977) to be largely reversible after five years antihypertensive treatment. In contrast similar changes of the calf muscles did not decrease significantly after six months treatment. In the present study the vascular distensibility of the resistance vessels in the anterior tibial muscle was equally reduced in untreated hypertensive patients and in patients having been well treated in the course of at least two years. Although the solution of this problem requires that a larger group of hypertensive subjects receiving effective antihypertensive treatment be studied, the previous and present findings indicate that the vascular structural changes of the leg muscle do not readily decline depite appropriate antihypertensive treatment. The possible difference in reversibility of the vascular changes in the upper and lower limbs might be explained by the different vascular transmural pressures in these two regions during active hours. Except when the supine body position is assumed the transmural pressure in the legs is higher than in the arms due to the higher hydrostatic pressure. Thus when the systemic blood pressure is reduced through antihypertensive treatment the relative decrease in the vascular transmural pressure will be smaller in the legs than in the arms.

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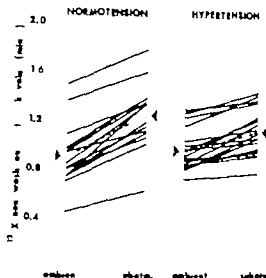


Fig. 2. The effect of exposing the leg to a subatmospheric pressure (subatm.) on the ¹³³Xenon wash-out rate from the anterior tibial muscle during reactive hyperemia in normotensive and untreated hypertensive subjects. The mean changes are indicated by the stippled lines.

during reactive hyperemia the resistance vessels are insensitive to vasoconstrictor drug (e.g. noradrenaline, angiotensine), even in high doses (Sivertsson 1970)

It might be argued that the difference in Δk between normotensive and hypertensive subjects could be explained by the very relationship between the vascular transmural pressure and the blood flow (cf Folkow and Löfving 1956) The curve formed by this relationship is concave to the pressure axis so that the increase in flow for a given increase in transmural pressure is smaller the higher the initial transmural pressure is. And as the transmural pressure is raised in hypertension because of the larger pressure head, a smaller flow increase could be expected. However this explanation does not satisfy the present findings since the increase in flow (Δk) was equally small in untreated and in well treated hypertensive patients, even though the pressure head of the latter was not different from that of the normotensive subjects. Moreover Hallbäck *et al* (1974) in perfusion studies on the hindquarters of normotensive and spontaneously hypertensive rats demonstrated that the resistance vessels of the hypertensive rats were always less distensible than those of the normotensive rats, even when the internal vascular radii and/or transmural pressures were initially the same in the two groups.

The finding that the resistance vessels exhibit a decreased response to stretching forces in a situation where their vascular smooth muscle cells can be assumed to be completely relaxed strongly indicates that the very structure of the vascular wall is changed in arterial hypertension. This was first suggested by Folkow and coworkers (1958), who demonstrated that in hypertensive subjects an increased thickness of the vessel wall in relation to the lumen might by itself account for the hyperreactivity to constrictor agents without necessarily implying an increased activity of the smooth muscle cells. This concept is further supported by later hemodynamic (Conway 1963 Folkow *et al* 1970, Sivertsson 1970, Folkow *et al* 1973) and morphological (Short 1966, Suwa and Takahashi 1971) investigations in man and animal

The vascular structural changes may be adaptive in nature but will probably contribute

Acta physiol. scand. 1978. 103. 437–445

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Observations on stretch reflexes in lumbar back muscles of the cat

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Received 25 January 1978

Abstract

CARLSON, H. *Observations on stretch reflexes in lumbar back muscles of the cat.* Acta physiol. scand. 1978. *103.* 437–445

Reflex responses to brief muscle stretch and to electrical stimulation of dorsal roots were studied in cat longissimus and iliocostalis muscles by myographic and electromyographic recordings in spinal and anaesthetized preparations. Brief stretch applied simultaneously to both muscles, by pulling at an isolated segment of the iliac bone, elicited contractions only in the central region of longissimus, composed of slowly contracting fibers, the time to peak tension varying between 70 and 100 ms. No reflex responses were observed in the faster contracting parts of this muscle or in the iliocostalis under the stimulation conditions used. The total reflex time usually varied from 4.5 to 7 ms which is shown to correspond to intraspinal conduction times, approximately between 2 and 5 ms. Only exceptionally was a central reflex delay corresponding to monosynaptic transmission observed. Reflexes evoked by dorsal root stimulation (L_6, L_7) had central conduction time similar to those elicited by adequate stimulation. The possibility that the central pathway for stretch reflexes in longissimus may involve more than two neurons is considered. In decerebrate preparations the central region of longissimus displays a prominent tonic stretch reflex. In longissimus it was seen the magnitude of the reflex is highly dependent on the position of the lumbar spine relative to the pelvic girdle.

The reflex response of an extremity muscle evoked by a brief muscle stretch, the myotatic reflex, is mediated through a two-neuron arc in which group I fibers originating from the muscle spindle form the afferent component of the pathway (for review see Matthews 1972). This type of proprioceptive reflex control is missing in certain muscles, as *e.g.* the facial muscles of the cat (Lindquist and Mårtensson 1970), the laryngeal muscles of the dog (Mårtensson 1963) and in the oculomotor muscles of the monkey (Keller and Robinson 1971). The two former muscular systems have no muscle spindles whereas the oculomotor muscles are supplied with spindles but do not respond to applied stretch.

At the spinal level, absence of monosynaptic reflexes in the dorsal muscles of the cat neck has been reported by Abrahams, Richmond and Rose (1975) even though the muscles are rich in spindles and the motoneurons receive monosynaptic excitation from low-threshold homonymous muscle afferents (Anderson 1977). However the reports give no information as to whether or not the dorsal neck muscles contract in response to adequate stimulation, *i.e.* stretch.

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Acta physiol. scand. 1978. 103. 437–445

From the Department of Physiology II, Karolinska Institutet, Stockholm, Sweden

Observations on stretch reflexes in lumbar back muscles of the cat

By

HANS CARLSON

Received 25 January 1978

Abstract

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Reflex responses to brief muscle stretch and to electrical stimulation of dorsal roots were studied in cat longissimus and iliocostalis muscles by myographic and electromyographic recordings in spinal and anemically decerebrated preparations. Brief stretch applied simultaneously to both muscles, by pulling at an isolated segment of the iliac bone, elicited contractions only in the central region of longissimus, composed of slowly contracting fibers, the time to peak tension varying between 70 and 100 ms. No reflex responses were observed in the faster contracting parts of this muscle or in the iliocostalis under the stimulation conditions used. The total reflex time usually varied from 4.5 to 7 ms which is shown to correspond to intraspinal conduction times, approximately between 2 and 5 ms. Only exceptionally was a central reflex delay corresponding to monosynaptic transmission observed. Reflexes evoked by dorsal root stimulation (L[illegible], L[illegible]) had central conduction times similar to those elicited by adequate stimulation. The possibility that the central pathway for stretch reflexes in longissimus may involve more than two neurons is considered. In decerebrate preparations the central region of longissimus displays a prominent tonic stretch reflex. With this preparation it was seen that the magnitude of the reflex is highly dependent on the position of the lumbar spine relative to the pelvic girdle.

The reflex response of an extremity muscle evoked by a brief muscle stretch, the myotatic reflex, is mediated through a two-neuron arc in which group I fibers originating from the muscle spindles form the afferent component of the pathway (for review see Matthews 1972). This type of proprioceptive reflex control is missing in certain muscles, as e.g. the facial muscles of the cat (Lindquist and Mårtensson 1970), the laryngeal muscles of the dog (Mårtensson 1963) and in the oculomotor muscles of the monkey (Keller and Robinson 1971). The two former muscular systems have no muscle spindles whereas the oculomotor muscles are supplied with spindles but do not respond to applied stretch.

At the spinal level, absence of monosynaptic reflexes in the dorsal muscles of the cat neck has been reported by Abrahams, Richmond and Rose (1975) even though the muscles are rich in spindles and the motoneurons receive monosynaptic excitation from low-threshold homonymous muscle afferents (Anderson 1977). However the reports give no information as to whether or not the dorsal neck muscles contract in response to adequate stimulation, i.e. stretch.

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The reflex response of an extremity muscle evoked by a brief muscle stretch, the myotatic reflex, is mediated through a two-neuron arc in which group I fibers originating from the muscle spindle form the afferent component of the pathway (for review see Matthews 1972). This type of proprioceptive reflex control is missing in certain muscles, as e.g. the facial muscles of the cat (Lindquist and Mårtensson 1970), the laryngeal muscles of the dog (Mårtensson 1963) and in the oculomotor muscles of the monkey (Keller and Robinson 1971). The two former muscular systems have no muscle spindles whereas the oculomotor muscles are supplied with spindles but do not respond to applied stretch.

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Thus, it is obvious that the presence of muscle spindles does not necessarily ensure a monosynaptic reflex activation of a muscle. A recent study of the cat's dorsal muscles in the lumbar region has shown that the muscles are supplied with spindles (Carlson 1978 b) but no evidence is available pointing to a monosynaptic transmission of reflexes in the longissimus and iliocostalis (cf. Carlson and Lindquist 1976). These two muscles form the main portion (about 80%) of the muscle mass in the lumbar back region, and since their contraction time is short (about 30 ms) they are well suited for quick phasic action. However, in a particular region of the longissimus there is a large proportion of slowly contracting fibers (contraction time about 100 ms) that can be selectively activated in reflexes evoked by dorsal root stimulation (Carlson 1978 a, b).

In the present investigation a closer analysis has been performed of reflex responses set up in the longissimus and iliocostalis muscles on application of adequate stimulation by brief and tonic stretch. The role of the longissimus in the control of the axial skeleton has also been analyzed.

Material and Methods

Preparations. Experiments were performed on 15 adult cats under ether anesthesia: 6 had their spinal cord transected through the dorsal atlanto-occipital membrane, 3 were decerebrated precollicularly and 6 were prepared for continued anesthesia by i.v. injections of chloralose (60–80 mg/kg b.wt.). At least 2 h was allowed for recovery after the ether anesthesia before the experiments started. Artificial respiration was instituted only to the preparations made spinal. The condition of the preparations was tested by studying the quadriceps and triceps surae reflex responses to tendon taps or to electrical dorsal root stimulation. Experimental data were collected from preparations in which these tests showed that stretch reflexes or monosynaptic reflexes could be obtained.

In spinal and anesthetized animals a midline skin incision was made, extending from the upper thoracic to the sacral region, and then the fascial layers in the lumbar region and the latissimus dorsi were cut through bilaterally. A laminectomy (L_4–L_7) was performed. The longissimus and iliocostalis muscles were separated from the iliac bone together with a small segment of the bone which was to be attached to the recording and pulling devices. The animals were fixed in a rigid frame as described in an earlier paper (Carlson 1978 a).

In experiments on decerebrate cats the muscle insertions were initially left intact so that the muscle length could be varied by manually changing the posture of the spine. The position of the head relative to the trunk was not affected by these manipulations. Reflex responses to sustained muscle stretch were also studied, applying procedures similar to those described for the other types of preparations.

The skin was sewn onto the frame to form a pool which was filled with liquid paraffin. The temperature in the pool was kept at 36–38°C by an infrared heating lamp.

Stimulation. In order to stretch the muscles a solenoid (Billman, type RD) was connected in series to an isometric strain gauge transducer (Grass FTO3) which was attached to the isolated segment of the iliac bone by a steel wire (Ø 0.6 mm). The solenoid was fed by a Grass S4 stimulator. Usually the amplitudes of the stretches ranged between 0.1 and 0.5 mm, but stretches of larger amplitudes (up to 4 mm) were also applied. The duration of the stretches was up to about 15 ms. The amplitudes and the durations of the stretches used in this study are within the same range as those used by e.g. Lloyd (1943) for elicitation of stretch reflex responses. More recent investigations of the spindle afferent activity in response to stretch have shown that primary endings from the cat's soleus muscle are activated by very small stretches (below 60 μm, Lundberg and Winsbury 1960) and that also secondary endings may discharge in response to small stretches (Stuart et al. 1970). In view of these results it appears reasonably safe to conclude that the endings of the back muscle spindles have responded to the stretch stimulus used.

For electrical stimulation of dorsal roots, square waves of 0.05–0.1 ms duration were delivered from a Grass S4 stimulator to bipolar silver-silver chloride wire electrodes.

Recordings. The arrangement with muscle, transducer and solenoid in series permitted recordings of the time course of the stretches as well as of the subsequent contractions. Electrical reflex responses were

recorded from the muscle with bipolar concentric needle electrodes (DISA®). The EMG method was used as it makes possible an accurate localization of reflexly activated muscle fibers within the muscle belly. Another factor favoring the use of EMG is the course of the back muscle nerves: since the thin branches run intramuscularly they are difficult to identify and liable to be damaged during the preparation. The reasons of the EMG for latency measurements will be evaluated in Results. Afferent activity in dorsal roots was recorded by pairs of silver–silver chloride wire electrodes.

The signals were fed into Grass P6 amplifiers via cathode followers, displayed on a Tektronix dual-beam oscilloscope and recorded by a Tandberg instrumentation recorder.

Results

Characteristics of reflex responses to brief muscle stretch and dorsal root stimulation in spinal and anesthetized preparations

The longissimus and iliocostalis muscles insert onto an intermuscular septum which runs along the middle and lower lumbar region and attaches to the iliac crest. Thus, pulling at an isolated segment of the iliac crest (*cf* Methods) results in a stretch of both muscles. In response to a brief stretch involving both muscles a reflex contraction is evoked as illustrated in Fig. 1 *A*. The lower record shows the time course of the stretch (initial upward deflection) as well as of the subsequent contraction (*cf* Methods) and the upper record represents the electrical response in the central part of the longissimus at the level of the 5th lumbar vertebra. From the figure the contraction time of reflexly activated motor units can be estimated: the time from the EMG response to peak contractile tension is about 70 ms. In the experiments contraction times of up to 100 ms have been recorded following activation by stretch or dorsal root stimulation (*cf* also Carlson 1978 a).

The amplitude of the EMG response was always largest in the central part of the longissimus along the middle and lower lumbar region as compared with other parts of this muscle and the iliocostalis. Even with larger amplitudes of muscle stretch (*cf* Methods) or when stretch was applied to the fascial layers of the back region in order to involve other portions of the muscles as well, no reflexes could be recorded in other parts than the central region of the longissimus.

The latency of the reflex in the longissimus in response to stretch appears from Fig. 1 *B*, in which the lower record shows the time course of the muscle stretch and the upper trace the EMG response. The latency of the electrical response from onset of stretch amounts to about 6 ms. The variation of the reflex latency in one and the same preparation was generally below 1 ms but the latency ranged from 4.5 to 7.0 ms in the different experiments. Only in one out of 10 preparations was a latency below 4 ms observed.

In order to determine the central delay of the reflex responses to stretch in the longissimus it was necessary to measure the time periods required for afferent and efferent conduction. By deafferentation of various segments it was found that the afferent loop of the reflex passes in the L_4 and L_5 dorsal roots. Since it has previously been shown that nerve fibers supplying slowly contracting longissimus muscle fibers run in the L_4 and L_5 ventral roots (Carlson 1978) it seems obvious that the reflex is mediated through these two segments.

The afferent volley recorded from whole dorsal roots or root filaments in response to stretch of the back muscles under study was small in comparison with the volley evoked by



Fig. 1 Reflex contraction of back muscles in response to brief stretch applied to longissimus and iliocostalis muscles. Upper traces. EMG recordings from central region of longissimus at level of 5th lumbar vertebra. Lower traces mechanical recordings of stretch (upward deflection) and reflex contraction (seen only in *A*). Time to peak tension in reflex contraction about 70 ms (*A*). Latency of reflex discharge from onset of stretch about 6 ms (*B*). Time bar in *A* 20 ms, in *B* ms.

a tap applied to the quadriceps tendon This may be due to the presence of only a small number of spindle afferents from the longissimus and iliocostalis muscles. In Fig. 2 *A* the upper record shows the afferent response in a filament of the 5th lumbar dorsal root and the lower record the time course of the muscle stretch A marked increase of the activity is seen about 1–2 ms after the onset of the stretch, which was a typical finding (*cf* below).

The efferent conduction time was measured by stimulating cut ventral roots and recording the electrical response within the muscles (*cf* Methods). A typical recording of such a response in the central region of the longissimus to stimulation of the 5th lumbar ventral root is illustrated in Fig. 2 *B* The time from the stimulus artefact to the first EMG deflection is about 1 4 ms. In the different experiments these values ranged from 1.3 to 1 7 ms. It may be argued that the latency of the EMG response is dependent on the recording site within the muscles it should be shorter in recordings near the end plate region than from more remote regions. In order to evaluate an error of this type latency measurements were made at high amplification and from different regions in the muscle. Since however the variations thus observed were small the conclusion can be drawn that a reasonably good precision was attained

Adding the time for the afferent conduction, about 1 0 ms, to that of the efferent conduction, about 1 4 ms, yields a total peripheral conduction time of about 2.4 ms. If the central conduction time for monosynaptic transmission, 1 1 ms according to Lloyd (1943), is added to this value the total reflex time should amount to about 3.5 ms. A latency in this range has occasionally been observed which indicates that monosynaptic reflex pathways do exist (*cf* above) As a rule, however as pointed out above, reflex responses to stretch had longer

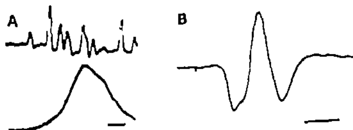


Fig. 2. *A* afferent volleys recorded from filament of L_4 dorsal root (upper trace) in response to stretch of longissimus (lower trace). Time from onset of stretch to first volley bout 1 ms. *B*, EMG recording from central region of longissimus in response to supramaximal stimulation of L_4 ventral root. Time from stimulus artefact to first EMG deflection 1.4 ms. Time bars 2 ms.

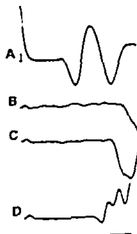


Fig. 3. EMG reflex discharge in central part of longissimus at level of 5th lumbar vertebra in response to dorsal root stimulation. *A*, L_5 dorsal root stimulation. *B–D*, from another experiment. In *B* and *C* stimulation applied to L_4 and L_5 dorsal root respectively. In *D* simultaneous stimulation of these roots. Note decrease in reflex latency when both roots are stimulated. Time bar 2 ms.

latencies, from 4.5 to 7.0 ms, which corresponds to central conduction times in the range from 2 to 5 ms.

The afferent volleys recorded from dorsal roots in response to stretch had a minimum latency of about 1 ms. This excludes the possibility that there should be a long delay between the onset of the stretch and the activation of afferent fibers which would contribute to a long total reflex time. However it might well be that the first afferent volley in response to stretch is subliminal and that the motoneurons do not discharge until on arrival of subsequent volleys. If this should be the case, there is a possibility that a synchronous afferent volley elicited by electrical stimulation of dorsal roots should give rise to reflexes of considerably shorter latencies than reflexes elicited by a muscle stretch.

A synchronous volley in the 5th lumbar dorsal root may occasionally elicit reflex discharge with a latency of about 2.5 ms (Fig. 3 *A*) but more common is a response of longer latency about 4.0 to 6.0 ms (Fig. 3 *B–D*). The recordings in *B–D* are all from the same experiment and illustrate the EMG response to stimulation of the L_4 root (*B*), the L_5 root (*C*) and to simultaneous stimulation (*D*). The latency of the reflex in *D* is about 1 ms shorter than that in *C* and 2 ms shorter than in *B*. Apparently spatial summation enhances the central reflex transmission. Orthodromic high-frequency stimulation over a brief period of time is known to potentiate the monosynaptic pathway (Lloyd 1949) to hind limb muscles. Such stimulation did not result in any remarkable change of the reflex in the longissimus muscle.

To sum up the experiments using adequate as well as those with electrical stimulation indicate that reflex responses recorded from the longissimus have only exceptionally a latency compatible with a monosynaptic reflex transmission. In most experiments a longer latency was recorded which appears to be due to a longer central delay

Demonstration of tonic stretch reflexes in decerebrate preparations

The central part of the longissimus exhibits prominent tonic stretch reflex. This appears from Fig. 4 in which the upper traces show EMG recordings from that part of the muscle

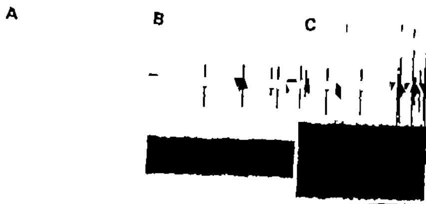


Fig. 4. EMG discharges (upper traces) from central region of longissimus at level of 5th lumbar vertebr[illegible] on stretch of muscle in steps of 2 mm (lower traces). Length in *A* shorter than the resting length. Tim[illegible] bar 100 ms.

at the level of the 5th lumbar vertebra and the lower traces represent the relative muscl[illegible] lengths. At a length shorter than the resting length (*A*) there is no activity while a recruit[illegible] ment of motor units occurs when the muscle is stretched in steps of 2 mm (*B C*) No suc[illegible] recruitment was observed in simultaneous recordings from peripheral parts of the long[illegible] issimus or from various regions of the iliocostalis.

The differentiation of the longissimus muscle into a central slowly contracting portion readily activated by steady stretch, and a peripheral fast contracting part with a high thresh[illegible] old for activation by maintained stretch is of interest from a functional point of view. In a previous investigation of the anatomy and contraction properties of back muscles th[illegible] longissimus was found to be able to control movements involving the pelvic girdle in rela[illegible] tion to the lumbar spine (Carlson 1978 a). The tonic stretch reflex can be utilized to obtain further information about the muscular control of the axial skeleton by studying its varia[illegible] tions in the intact muscle when the spine is passively brought into different postures affecting the muscle length. As demonstrated in the previous paper the mobility of each lumbar intervertebral joint is smaller than that of the lumbosacral joint. When the latter joint is passively flexed, resulting in a stretch of the longissimus, a tonic activity in the centra[illegible] region is set up or increased. Conversely when the joint is passively extended the tonic activity invariably decreases or ceases. This is illustrated in Fig. 5 *A* showing EMG responses obtained during flexion (upper trace) and extension (lower trace) of the lumbosacral joint. In these positions of the joint the peripheral parts of the longissimus as well as the iliocostalis appear to be inactive. A change of the position in the vertical plane in other lumbar joints proved to be less effective in altering the degree of the tonic activity in the central part of the muscle. This portion of the muscle thus seems to be specifically engaged in the tonic control of the lumbosacral joint position or in other terms, in the control of the posture of the lower lumbar spine versus the pelvic girdle. A stretch of the muscle is brought about by flexion in the joint and elicits a reflex contraction opposing the intended movement.

A muscle stretch occurs also on a horizontal bending of the spine, and this elicits a reflex response in the central part of the muscle in the same way as does a vertical bending. The mobility of the lumbar vertebral column in the horizontal plane is small but evidently the

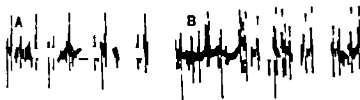


Fig. 5. EMG activity in central longissimus at level of 5th lumbar vertebra when axial skeleton is forced into different postures. *A*, upper record, lumbosacral joint flexed; lower record, same joint extended. *B*, simultaneous recordings from muscles at both sides when lower lumbar spine is bent toward one side in the horizontal plane. Marked activity in contralateral muscle (upper trace), ipsilateral muscle (lower trace) silent. Time bar 100 ms.

muscle stretch thus induced is large enough to activate the muscle. In horizontal bendings of the spine the longissimus muscles at both sides display a reciprocal activity pattern. When the lower lumbar region is passively bent toward one side a tonic activity is set up in the contralateral muscle, as illustrated in the upper trace of Fig. 5 *B*, whereas the ipsilateral muscle is inactive (lower trace). This implies that the muscle at one side should be capable to bend the spine toward the same side when the pelvic bone is fixed.

The experiments thus show that the central regions of the longissimus muscles at both sides of the spine act in synergy when vertical flexions of the lowest part of the spine are to be counteracted, whereas they act as antagonists in horizontal bendings.

Discussion

The results presented in this study indicate that a selective activation of slowly contracting motor units in the central part of the longissimus muscle occurs in response to brief as well as to maintained stretch. No reflex responses have been obtained from the faster contracting parts of the longissimus or from the iliocostalis (contraction times about 30 ms, *cf.* Carlson 1978 a). This selective reflex activation may be due to several factors but one obvious cause may be the different distribution of muscle spindles, the central part of the longissimus holding more spindles than other regions of the two muscles (Carlson 1978 b).

The central region of the longissimus is made up of a high proportion of fibers which stain light for myofibrillar ATPase (type I) whereas fibers staining darkly for the ATPase (type II) are predominant in the peripheral longissimus as well as in the whole iliocostalis. A similar uneven distribution of various fiber types is present in some extremity muscles, e. g. the quadriceps and the tibialis anterior and differences in reflex thresholds have been observed for different regions within these muscles (Denny-Brown 1929, Gordon and Phillips 1953). As demonstrated by Henneman *et al.* (1965) and by Burke (1968), the small slow-twitch units in limb muscles have the lowest threshold for reflex activation by stretch. It is evident that the longissimus together with the iliocostalis forms a muscular system consisting of a small, slowly contracting component with a low reflex threshold for stretch and a large, fast contracting component which is not recruited by the stretch stimuli used in the present investigation.

In the previous study of the morphology and contraction properties of the back muscl the longissimus and the iliocostalis were found to be involved in the control of the positic of the lumbar spine versus the pelvic girdle (Carlson 1978 a) The effects observed whe stretching the longissimus muscle in the decerebrate preparation by forcing the lower spi into different postures suggest that the slowly contracting component of the longissimu may be specifically engaged in the tonic control of the vertical position of the lumbosacr joint. This part of the muscle also exerts a tonic control of horizontal bendings in the low lumbar spine.

It is generally accepted that the reflex response to brief stretch in extremity muscles set up by monosynaptic excitation from Ia afferents. A monosynaptic pathway from primar spindle afferents does probably also exist for the longissimus since appropriate latenci have been observed experimentally on application of adequate as well as electrical stimula tion. A reflex transmission through such a pathway seems however to be exceptional.

As mentioned in the introduction this appears to be the case also as far as the dors muscles in the neck region are concerned, as shown by Abrahams, Richmond and Ro (1975) In a pool of 96 motoneurons supplying these muscles only one could be activate by dorsal root stimulation with a central delay shorter than 2 ms at a stimulus strengt sufficient for activation of group I and II fibers 26 neurons were activated after 7–25 m Their analysis offers no information about dorsal neck muscle reflexes in response t stretch Recent experiments (Carlson and Skoglund, unpublished) indicate that these mu cles are activated by a brief muscle stretch with a latency in the same range as that usu ally observed for the longissimus muscle.

In most experiments the shortest central delay of reflexes in the longissimus elicited b stretch as well as by electrical stimulation was long enough to give time for transmissio through a polysynaptic pathway In flexors of the hind limb autogenetic excitation can be set up from secondary endings (*cf* Laporte and Bessou 1959 Eccles and Lundberg 1959) and a similar mechanism might be responsible for the reflex activation of the longissimu muscle. Such excitatory reflex effects may via polysynaptic routes, add to a subthreshold monosynaptic Ia excitation and cause a discharge of the motoneurons after a longer delay than that of a monosynaptic reflex. However an activation of other sense organs by brief stretch is of course plausible, and the possibility that the reflex discharges observed are due to the central action of other receptors than the spindles (*cf* Paintal 1960) can therefore not be entirely excluded.

Another mechanism that must be taken into consideration is reflex activation by poly-synaptic autogenetic group Ia excitation (for ref see Homma, 1976) Some evidence for excitatory synaptic actions on hind limb motoneurons by such a mechanism has recently been presented by Taylor *et al* (1976). Reflex discharges after very long latencies (50–80 ms) following stimulation of hind limb Ia afferents have been demonstrated by Hultborn, Wig-ström and Wängberg (1975) As far as the central reflex mechanisms of lumbar back muscles are concerned it is not possible to draw any definite conclusions at the present stage. Further experimentation, preferably using intracellular recordings from the back muscle moto-neurons, is required.

This work was supported by a grant from the funds of Karolinska Institutet.

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In the previous study of the morphology and contraction properties of the back muscl the longissimus and the iliocostalis were found to be involved in the control of the postu of the lumbar spine versus the pelvic girdle (Carlson 1978 a) The effects observed wh stretching the longissimus muscle in the decerebrate preparation by forcing the lower sp into different postures suggest that the slowly contracting component of the longissim may be specifically engaged in the tonic control of the vertical position of the lumbosacr joint. This part of the muscle also exerts a tonic control of horizontal bendings in the low lumbar spine.

It is generally accepted that the reflex response to brief stretch in extremity muscles set up by monosynaptic excitation from Ia afferents. A monosynaptic pathway from prima spindle afferents does probably also exist for the longissimus since appropriate latenci have been observed experimentally on application of adequate as well as electrical stimul tion *A reflex transmission through such a pathway seems however to be exceptional.*

As mentioned in the introduction this appears to be the case also as far as the dors muscles in the neck region are concerned as shown by Abrahams, Richmond and Ro (1975). In a pool of 96 motoneurons supplying these muscles only one could be activate by dorsal root stimulation with a central delay shorter than 2 ms at a stimulus strengt sufficient for activation of group I and II fibers 26 neurons were activated after 7–25 m Their analysis offers no information about dorsal neck muscle reflexes in response t stretch Recent experiments (Carlson and Skoglund, unpublished) indicate that these mu cles are activated by a brief muscle stretch with a latency in the same range as that us ally observed for the longissimus muscle.

In most experiments the shortest central delay of reflexes in the longissimus elicited b stretch as well as by electrical stimulation was long enough to give time for transmissio through a polysynaptic pathway In flexors of the hind limb autogenetic excitation can b set up from secondary endings (*cf* Laporte and Bessou 1959 Eccles and Lundberg 1959) and a similar mechanism might be responsible for the reflex activation of the longissimu muscle. Such excitatory reflex effects may via polysynaptic routes, add to a subthreshol monosynaptic Ia excitation and cause a discharge of the motoneurons after a longer dela than that of a monosynaptic reflex. However an activation of other sense organs by brief stretch is of course plausible, and the possibility that the reflex discharges observed are due to the central action of other receptors than the spindles (*cf* Paintal 1960) can therefore not be entirely excluded

Another mechanism that must be taken into consideration is reflex activation by poly-synaptic autogenetic group Ia excitation (for ref see Homma, 1976) Some evidence for excitatory synaptic actions on hind limb motoneurons by such a mechanism has recently been presented by Taylor *et al* (1976). Reflex discharges after very long latencies (50–80 ms) following stimulation of hind limb Ia afferents have been demonstrated by Hultborn Wig ström and Wängberg (1975). *As far as the central reflex mechanisms of* lumbar back muscles are concerned it is not possible to draw any definite conclusions at the present stage. Further experimentation, preferably using intracellular recordings from the back muscle moto-neurons, is required

This work was supported by a grant from the funds of Karolinska Institutet.

[illegible] of work. Thus at submaximal intensities (30–85% $\dot{V}O_2$ max) the loss of glycogen [illegible] mainly in type I fibres, whereas type II fibres are depleted only when the exercise is prolonged (Gollnick *et al.* 1973, Gollnick, Piehl and Saltin 1974). With intense work loads a glycogen depletion is found in both type I and type II fibres, although the depletion pattern [illegible] with the mode of work. Thus a difference between intermittent and continuous [illegible] exercise has been described, in that during intermittent exercise the glycogen depletion is more pronounced in fibres with the darker NADH-dehydrogenase-stain [illegible] *i.e.* those with a higher oxidative capacity (Edgerton *et al.* 1975)

An estimation of glycogen depletion by a histochemical technique is at the best semi-quantitative and detailed quantitative information cannot be obtained as differences in the PAS-staining intensity above 80 mmol/kg wet weight are difficult to detect (Piehl 1974). The purpose of the present study was therefore to investigate, in greater detail by quantitative analyses, the glycogen content in type I, II A and II B fibres at rest, as well as the utilization of glycogen in these fibres during various types of exercise. A comparison was [illegible] made of glycogen depletion during intermittent and continuous exercise at high work intensities with continuous exercise at moderate work intensity

Subjects

[illegible] healthy volunteers participated in the study. Mean values and ranges were for: age 24 (21–41) years, height 179 (174–193) cm, weight 73 (62–87) kg and maximal oxygen uptake 3.89 (2.92–4.21) l/min. All [illegible] were well informed about the procedures to be used before giving their oral consent to participate in the study

Procedure and Methods

Maximal oxygen uptake during bicycling at 60 rpm for the 13 subjects was determined in [illegible] preparatory test. All expts. were performed on [illegible] Siemens-Elema bicycle ergometer in the sitting position.

In order to analyse the glycogen depletion in the two main fibre types (I and II), the following expts. were performed. 5 subjects performed intermittent intense exercise (15 [illegible] work 15 [illegible] rest) for 60 min at the work intensity [illegible] which demanded the subject's *maximal oxygen uptake during continuous exercise.* At [illegible] later [illegible] in the laboratory the same 5 subjects performed continuous submaximal exercise for 60 min at approximately half the [illegible] work intensity used in intermittent exercise. Oxygen uptake was determined after 15, 30 and [illegible] min duration in all situations. Data on overall substrate utilization from these expts. have been presented previously (Essén, Hagenfeldt and Kaijser 1977)

In order to analyse the glycogen depletion not only in type I and II fibres but also in the subgroups of type II fibres, some additional similar experiments were performed. 3 subjects performed intermittent intense exercise for 60 min and 2 subjects continuous submaximal exercise for 60 min. Finally 3 subjects performed continuous maximal exercise at [illegible] similar work intensity to that in the experiments with intermittent exercise, this led to exhaustion after 4–6 min.

Muscle biopsies from the vastus lateralis were obtained at rest and at the end of all the expts. and in some subjects also after 5 and 30 min duration of the exercise.

Analyses. Expired air was collected in Douglas bags. The volume of the air was measured in [illegible] spirometer and was analysed for the contents of O_2 and CO_2 with the Haldane technique. Muscle samples were obtained with [illegible] percutaneous biopsy technique (Bergström 1962) and were frozen immediately in liquid nitrogen and then stored at [illegible] 80°C until analysed. After freeze-drying of the muscle samples, individual fibre fragments were dissected out (100–200 fibres from each sample). In order to identify the fibre fragments, 2 3 small pieces were cut off each one and stained for myofibrillar ATPase after preincubation at pH 10.3 4.6 or 4.3 (Essén *et al.* 1975) The remaining part of the fibre was weighed on [illegible] quartz fibre balance, and its glycogen hydrolysed in 1 M HCl at 100°C for 2 h. The samples were then cooled and aliquots were taken for subsequent analyses of glucose residues (Essén and Henriksson 1975).

Acta physiol. scand. 1978. 103. 446–455

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Glycogen depletion of different fibre types in human skeletal muscle during intermittent and continuous exercise

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Received 26 January 1978

Abstract

Essén B Glycogen depletion of different fibre types in human skeletal muscle during intermittent and continuous exercise Acta physiol scand. 1978 103 446–455

Muscle biopsies from the vastus lateralis were obtained from thirteen healthy subjects at rest and at inten during continuous and intermittent exercise. After freeze-drying of the muscle sample, fragments of sing fibres were dissected out and stained for myofibrillar ATPase with preincubation at pH 10.3, 4.6 or 4.3 t identify type I II A and II B fibres respectively The remaining part of each fragment was used for quar titative glycogen analyses. At rest, mean glycogen content was significantly higher in type II fibres (40 mmol/kg dry weight) than in type I (344 mmol/kg dry weight). After 60 min continuous exercise at moderat work load a more pronounced glycogen depletion had occurred in type I (277 mmol/kg dry weight) than i type II (A + B) fibres (113 mmol/kg dry weight). With 60 min intense intermittent exercise a significant as similar depletion occurred in both type I (213 mmol/kg dry weight) and type II (A + B) fibres (203 mmol/k dry weight). With continuous intense exercise to exhaustion (4–6 min), glycogen depletion was more marke in type II (A + B) (118 mmol/kg dry weight) than type I fibres (74 mmol/kg dry weight). These data impl that the glycogen depletion pattern in muscle fibres is determined mainly by the work intensity but the low glycogen depletion per unit time in intermittent compared with continuous tense exercise indicates tha the mode and duration of exercise is important, too.

Glycogen utilization in human skeletal muscle varies with the intensity duration and mode of exercise, which to some extent seems to be due to a pattern of selective recruitment of muscle fibres with different metabolic characteristics. Based on stainings for myofibrillar ATPase activity the fibres of human skeletal muscle can be classified into two main groups, type I and II (Engel 1962) Type I fibres usually have the higher oxidative capacity type II fibres the higher glycolytic capacity (Dubowitz and Brooke 1973). Type II fibres can be subclassified into type II A and II B (Brooke and Kaiser 1970), the former apparently having the higher oxidative capacity and the latter the higher glycolytic capacity (Essén *et al* 1975). In spite of these differences in glycolytic potential, no systematic quantitative difference in basal glycogen content has been established between the different fibre types in man.

During exercise, glycogen seems to disappear at different rates in the different fibre types based on histochemical data and these differences are dependent on both load and

TABLE II Glycogen content (mean ± S.D.) in type I, II A and II B fibres at rest and after 60 min intermittent intensive exercise (273 watt) and continuous submaximal exercise (160 watt).

Subject	Fibre type distribution			Intermittent intensive exercise (15 s work 15 s rest)											
				Rest			5 min			30 min			60 min		
	I	II A	II B	I	II A	II B	I	II A	II B	I	II A	II B	I	II A	II B
6	56	23	21	378 ±138 (25)	407 ±119 (79)	467 ±79b (17)	346 ±90d (52)	324 ±94d (40)	311 ±93d (19)	219 ±112d (39)	234 ±105d (36)	215 ±98d (12)	117 ±103d (79)	183 ±137a (48)	226 ±143b (24)
7	45	45	10	388 ±106 (39)	386 ±123 (20)	396 ±95 (8)	334 ±100d (26)	376 ±88 (56)	375 ±145 (16)	182 ±57d (68)	287 ±63a (46)	253 ±64b,d (38)	92 ±71d (51)	169 ±139a (39)	119 ±72d (16)
8	50	35	15	348 ±99 (52)	368 ±99 (50)	398 ±70d (28)	—	—	—	—	—	—	141 ±75d (86)	255 ±181a,d (75)	304 ±176b (47)
Continuous submaximal exercise															
9	64	28	8	413 ±76 (35)	464 ±86 (34)	483 ±54 (10)	346 ±58d (38)	478 ±52a (26)	467 ±82b (11)	—	—	—	69 ±45d (85)	435 ±127a (59)	289 ±141b,d (21)
10	18	50	32	513 ±70 (32)	589 ±87 (25)	640 ±73b (17)	—	—	—	—	—	—	84 ±5c,d (38)	345 ±199a,d (33)	466 ±121b,c (16)

a Higher mean value in type II A than type I fibres ($p < 0.05$).
b Higher mean value in type II B than type I fibres ($p < 0.05$).
c Higher mean value in type II B than type II A fibres ($p < 0.05$).
d Lower mean value than at rest ($p < 0.05$).
Numbers within parentheses denote the number of fibres analysed for glycogen.

In order to establish the percentage of each fibre type in each subject, part of a muscle sample was prepared for histochemical analyses and serial cross sections were stained for myofibrillar ATPase after acid and alkaline preincubation (Brooke *et al.* 1970).

Statistics. The significance of differences between categories was determined according to Student's *t*-test and the rank sum test (Dixon and Massey 1957).

Results

Work intensity

In intermittent exercise, mean work intensity and oxygen uptake were 299 (range 270–343) W and 2.21 (1.93–2.81) l/min, respectively. In continuous submaximal exercise they were 157 (138–180) W and 2.31 (1.93–2.84) l/min, and in continuous maximal exercise 266 (220–300) W and 3.56 (2.92–3.94) l/min. Mean oxygen uptake values corresponded to 50–60 % of the subject's maximal oxygen uptake ($\dot{V}O_2$ max) in both intermittent intense and continuous submaximal exercise, whereas the corresponding value during continuous maximal exercise was 100% $\dot{V}O_2$ max.

Glycogen content at rest (Table I–III)

At rest a large variation in glycogen content was found in all subjects within type I, II A and II B fibres respectively and the values were distributed symmetrically around the mean (Fig. 1 and 2). The mean concentration was higher in type II than in type I fibres in all but one subject. The mean value and range for type I fibres, 344 (223–513) mmol/kg dry weight, was significantly lower ($p < 0.001$) than in type II fibres, 402 (271–610) mmol/kg dry weight. No significant difference was found between the mean glycogen content of type II A and type II B fibres, 430 (335–589) and 454 (350–640) mmol/kg dry weight.

TABLE I. Glycogen content (mean ± S.D.) in type I and II fibres at rest and after 60 min intermittent (15 s work–15 s rest) intense exercise (299 watt) and continuous submaximal exercise (157 watt).

Subject	Fibre type distribution %			Intermittent intense exercise (15 s work–15 s rest)				Continuous submaximal exercise			
				Rest		60 min		Rest		60 min	
	I	II A	II B	I	II	I	II	I	II	I	II
1	63	33	4	292±75 (28)	377±79[a] (37)	120±101[b] (29)	252±123[b] (52)	288±70 (47)	404±91 (44)	95±130[b] (28)	362±199 (28)
2	57	32	11	433±121 (37)	441±102 (60)	89±72[b] (33)	187±105[b] (69)	303±64 (57)	179±89[a] (41)	36±60[b] (68)	251±102[b] (34)
3	49	45	6	283±64 (29)	271±44 (60)	46±65[b] (33)	39±56[b] (73)	400±57 (74)	407±70 (62)	94±99[b] (91)	189±111[b] (76)
4	52	38	10	250±53 (41)	344±78[a] (58)	197±100[b] (110)	109±91[b] (107)	360±64 (45)	381±73 (52)	131±98[b] (73)	388±1.2 (65)
5	58	29	13	279±81 (20)	302±56 (75)	148±80[b] (76)	96±81[b] (109)	355±79 (39)	427±105[a] (43)	184±164[b] (46)	320±117[a] (64)

[a] Higher mean value in type II than type I fibres ($p < 0.05$).
[b] Lower mean value than at rest ($p < 0.05$).
Numbers within parentheses denote the number of fibres analysed for glycogen.

TABLE II. Glycogen content (mean ± S.D.) in type I, II A and II B fibres at rest and after 60 min intermittent intense exercise (273 watt) and continuous submaximal exercise (160 W).

Subject	Fibre type distribution			Intermittent intense exercise (15 s work, 15 s rest)											
				Rest			5 min			30 min			60 min		
	I	II A	II B	I	II A	II B	I	II A	II B	I	II A	II B	I	II A	II B
6	56	23	21	370 ±138 (25)	407 ±119 (29)	467 $\pm 79^{b}$ (17)	346 $\pm 90^{d}$ (52)	326 $\pm 94^{d}$ (40)	311 $\pm 93^{d}$ (19)	219 $\pm 112^{d}$ (39)	234 $\pm 105^{d}$ (34)	215 $\pm 98^{d}$ (12)	117 $\pm 101^{d}$ (79)	183 $\pm 137^{a}$ (48)	226 $\pm 143^{b}$ (24)
7	45	45	10	388 ±106 (39)	386 ±123 (20)	396 ±93 (8)	334 $\pm 100^{d}$ (24)	376 ±88 (56)	373 ±145 (16)	182 $\pm 57^{d}$ (68)	287 $\pm 63^{a}$ (46)	233 $\pm 64^{b}$ (38)	92 $\pm 71^{d}$ (31)	169 $\pm 135^{a}$ (39)	119 $\pm 72^{d}$ (16)
8	50	35	15	348 ±99 (52)	368 ±99 (50)	398 $\pm 70^{a}$ (28)	—	—	—	—	—	—	141 ±73 (86)	255 $\pm 181^{a,d}$ (75)	304 $\pm 176^{b,d}$ (47)
Continuous submaximal exercise															
9	64	28	8	413 ±76 (35)	464 $\pm 86^{a}$ (34)	483 $\pm 54^{b}$ (10)	346 $\pm 38^{d}$ (38)	478 $\pm 52^{a}$ (26)	467 $\pm 82^{b}$ (11)	—	—	—	69 $\pm 45^{d}$ (85)	433 $\pm 127^{a}$ (59)	289 ±141 (21)
10	18	50	32	513 ±70 (32)	589 ±87 (25)	640 $\pm 73^{b}$ (17)	—	—	—	—	—	—	84 $\pm 57^{d}$ (38)	345 $\pm 199^{a}$ (33)	466 $\pm 121^{b,c}$ (16)

[a] Higher mean value in type II A than type I fibres ($p < 0.05$).
[b] Higher mean value in type II B than type I fibres ($p < 0.05$).
[c] Higher mean value in type II B than type II A fibres ($p < 0.05$).
[d] Lower mean value than at rest ($p < 0.05$).
Numbers within parentheses denote the number of fibres analysed for glycogen.

TABLE III Glycogen content (mean ± S.D.) in type I, II A and II B fibres at rest and at end of (4–5 min) continuous intense exercise to exhaustion (267 watt).

Subject	Fibre type distribution %			Continuous intense exercise to exhaustion					
				Rest			Exercise (4–6 min)		
	I	II A	II B	I	II A	II B	I	II A	II B
11	35	50	15	223 ± 131 (44)	335 ± 142^{a} (61)	350 ± 121^{b} (2[illegible])	137 ± 45^{d} (24)	$46 \pm 48^{a,d}$ (36)	$207 \pm 54^{b,d}$ (24)
12	61	30	9	409 ± 79 (23)	470 ± 106 (18)	515 ± 76^{b} (9)	359 ± 70^{d} (50)	$396 \pm 82^{a,d}$ (30)	$441 \pm 60^{b,c,d}$ (14)
13	73	25	2	284 ± 71 (41)	419 ± 60^{a} (37)	383 ± 43^{b} (14)	199 ± 70^{d} (99)	$239 \pm 88^{a,d}$ (47)	$40 \pm 49^{b,d}$ (7)

[a] Higher mean value in type II A than type I fibres ($p < 0.05$).
[b] Higher mean value in type II B than type I fibres ($p < 0.05$).
[c] Higher mean value in type II B than type II A fibres ($p < 0.05$).
[d] Lower mean value than at rest ($p < 0.05$).
Numbers within parentheses denote the number of fibres analysed for glycogen.

Glycogen depletion during continuous submaximal exercise (Table I, II)

At the end of the continuous submaximal exercise a mean decrease of 277 (171–344) mmol/kg dry weight ($p < 0.001$) was found in type I fibres and 113 (−7–225) mmol/kg dry weight ($p < 0.05$) in type II fibres (Fig. 1). The depletion was more pronounced in type I than in type II fibres ($p < 0.01$). The pattern for glycogen content in the different fibre types, as shown in Fig. 1 indicates that most of the type I fibres had lost glycogen with this mode of exercise but only some of the type II fibres.

The two subjects in whom glycogen was analysed separately in type II A and II B fibres showed great variations in their fibre type distribution as well as in their glycogen depletion pattern (Table II). Thus the subject with 64% type I fibres showed a significant decrease of glycogen in these fibres as well as in the few type II B fibres, while no decrease was seen in type II A fibres after 60 min. The other subject, who had only 18% type I fibres, showed a significant decrease in glycogen in all 3 types of fibres after 60 min.

Glycogen depletion during intermittent intense exercise (Tables I, II)

After 60 min intermittent exercise the mean decrease was in type I fibres 213 (53–344) mmol/kg dry weight ($p < 0.001$) and in type II fibres 203 (125–254) mmol/kg dry weight ($p < 0.001$). In the 3 subjects where glycogen was analysed in the subgroups of type II fibres, a decrease had occurred at the end of exercise in both type II A and II B fibres within each subject ($p < 0.05$) (Fig. 2). The mean decrease was in type II A 185 (113–224) mmol/kg dry weight and in type II B 204 (94–277) mmol/kg dry weight.

Glycogen depletion during continuous intense exercise to exhaustion (Table III)

A significant glycogen decrease was found in type I, II A and II B fibres in each of the 3 subjects who performed continuous exercise to exhaustion. The mean decreases in the 3

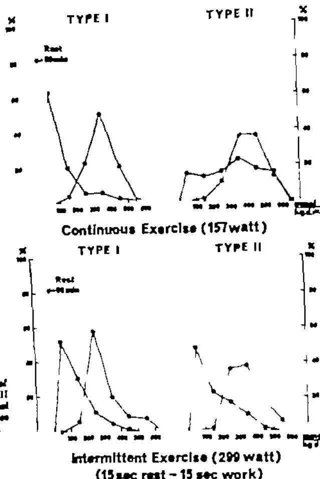


Fig. 1. The distribution pattern of glycogen content in type I and II fibres with continuous submaximal exercise and intermittent intense exercise (mean from 5 subjects).

types were 74 (50–86), 114 (74–180) and 120 (74–143) mmol/kg dry weight respectively. In all 3 subjects the glycogen decrease was greater in type II than in type I fibres. The distribution pattern of glycogen content in the different fibre types indicates that within each fibre type most fibres had lost some glycogen with intense exercise (Fig. 2).

The glycogen depletion in type II A fibres, was 2 times greater for the subject with the lowest percentage of these fibres (25%) than in the subjects with the highest (50%).

Discussion

Quantitative analyses in single fibre fragments showed at rest a slightly greater mean glycogen content in type II than in type I fibres. With continuous prolonged exercise for 60 min at moderate work intensity glycogen depletion was more pronounced in type I than in type II fibres. At high work intensity performed either as continuous or as intermittent exercise, both type I and type II fibres were glycogen depleted. The intermittent intense

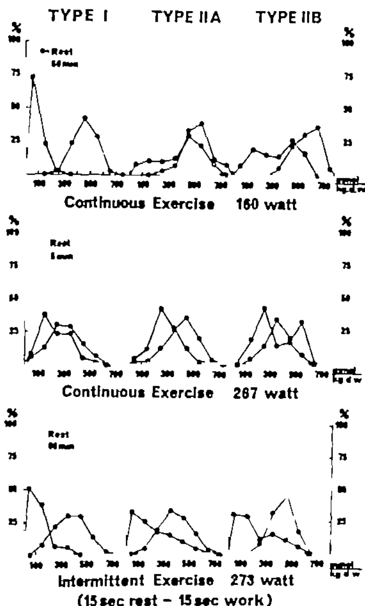


Fig. .. The distribution pattern of glycogen content in type I, II A and II B fibres with continuous submaximal exercise (mean of subjects), continuous intense exercise (mean of 3 subjects) and intermittent intense exercise (mean of 3 subjects).

exercise, however, gave a smaller depletion per unit time than continuous intense exercise. The data thus imply that the glycogen depletion pattern of muscle fibres during bicycle exercise is determined mainly by the work intensity but that the mode and duration of exercise is evidently of importance, too.

Glycogen content at rest

The slightly greater mean resting glycogen content in type II than type I fibres seems to indicate that type II fibres normally have the greater capacity for glycogen storage. However, it is worth emphasizing that the ranges of glycogen content for both the type I and the type II fibre population, are wide and overlapping. When evaluating the initial level of glycogen in different fibre types it must also be remembered that glycogen depletion

already occurs preferentially in the type I fibres at the work intensities associated with daily routine activities. In this study the subjects were all studied in the morning and no intense physical exercise had been performed before the expt. However even physical activity and diet the day before the expt. might be of significance, as it is known that glycogen repletion can be a fairly slow process (Piehl 1974).

Glycogen depletion during continuous submaximal exercise

The marked glycogen depletion in type I fibres and the slight depletion in type II fibres in most subjects after 60 min submaximal exercise is in good agreement with earlier findings using histochemical techniques (Gollnick *et al.* 1973 Gollnick *et al.* 1974). The distribution pattern at rest and at the end of exercise for fibres with different glycogen contents indicates that all of the type I fibres have been involved during submaximal continuous exercise, but only some of the type II fibres. Glycogen depletion in the subgroups of type II fibres has previously been studied, only at higher work intensities (~85% VO max), using histochemical analysis, which has shown that type II A fibres are more depleted than type II B fibres (Andersen and Sjøgaard 1975). The present study shows that in addition to a marked depletion of type I fibres, both type II A and type II B fibres to some extent can be glycogen depleted even at lower work intensities (~50–60% VO_2 max). The magnitude of this depletion seems to be dependent on the subject s percentage distribution of fibre types, as discussed below

Glycogen depletion during continuous intense exercise

With maximal exercise (~100% $\dot{V}O_2$ max) glycogen depletion occurred in all three fibre types, suggesting that all these fibres had been activated during some phase of the work. The depletion was more marked in type II than type I fibres. One factor which might contribute to this difference is that type II fibres have a greater glycolytic capacity than type I fibres. Histochemical analyses have also shown a glycogen depletion in both type II and type I fibres, more marked in type II, although those studies were performed at work intensities of 120–150% VO_2 max (Gollnick *et al* 1973 1974).

Glycogen depletion during intermittent intense exercise

The overall metabolic response in intermittent intense exercise is found to be more similar to continuous exercise of lower intensity than to continuous exercise of the same high work intensity (Åstrand *et al.* 1960, Essén *et al* 1977). The present study indicates that during intermittent intense exercise, type I II A and II B fibres show a glycogen depletion pattern which is more comparable to that of continuous maximal exercise than continuous submaximal exercise (Fig 1 2). However with intermittent exercise, glycogen depletion per unit time was lower than with continuous maximal work in both type I and type II fibres and usually of a similar magnitude. These findings seem to indicate that the glycogen depletion pattern is determined mainly by the work intensity although the rate of glycogen depletion is influenced by the mode of exercise, in both fibre types.

The lower glycogen depletion in intermittent exercise may then be explained by a relative increase in lipid contribution to oxidative metabolism, which is suggested to be due to the

increased levels of ATP CP and citrate at the end of each rest period, thus suppressing glycolysis in the early phase of the subsequent work period (Essén et al. 1977) There is also a possible higher oxygen availability due to reloading of myoglobin stores in the resting periods, and subsequently a greater aerobic energy output which gives a higher ATP production per glucose unit as compared to lactate formation

Glycogen depletion and fibre type distribution in muscle

Subjects with low percentage type I fibres showed different glycogen depletion pattern than those with low percentage type II fibres. Thus with continuous submaximal exercise the subject with a low percentage of type I fibres showed a greater depletion in type II fibres than the subject with a greater percentage of type I fibres, probably because the tension development exceeded the capability of type I fibres already at low work load. With continuous intense exercise the subject with a low percentage of type II fibres showed a greater glycogen depletion in these fibres than the subject with twice as many type II fibres, which indicates that each one of these fibres is involved more often at higher work intensities. The involvement of type II fibres is probably due to the rapid tension development at the start of each pedal thrust and the fact that peak tension amounts to a significant fraction of maximal voluntary contraction, which cannot be achieved by type I fibres alone, as has been shown for submaximal continuous exercise (85% VO max) (Andersen et al 1975)

Glycogen depletion pattern and fibre recruitment

Glycogen depletion indicates that the fibres have been active during some phase of a work bout, contributing to the tension development. However no exact information can be obtained about the extent of fibre activation as several other substrates than glycogen are available in the muscle cell, such as glucose, fatty acids, and triglycerides these are probably used at different rates depending on the work intensity duration of exercise and availability of substrates. This contribution from other substrates may differ between fibre types. As stated above, intermittent intense exercise is a good example of situations where these substrates are of great importance, thus contributing to a glycogen saving effect. Other circumstances which limit the use of glycogen depletion data as an indication of fibre recruitment are that glycogen with complete combustion gives a greater energy release than with lactate formation, that glycolysis might occur without tension output, and that glycogen resynthesis may occur in the muscle cell However with all these limitations in mind, glycogen depletion over a substantial period indicates that the fibres have contributed to the total tension output and can thus be taken as an index for fibre recruitment.

A preferential recruitment of type I fibres would thus occur at low work intensity in contrast to recruitment of both type I and type II fibres at higher work intensities. This statement is consistent with the innervation pattern found in the cat where type I fibres are innervated by small motor neurons with low activation thresholds, whereas motor units with larger motor neurons and high thresholds innervate type II fibres (Henneman and Olson 1965)

During intermittent intense exercise, type II fibres involvement may be due not only to

the intense activity but also to the repeated phases that occur of increased load to accelerate from stand still to 60 rpm with each work period. It has been shown in voluntary contraction of the toe extensor muscle, with rapid acceleration phases, that motor units with type II fibres are preferentially recruited before those with type I fibres (Grimby and Hannerz 1977).

In conclusion, glycogen depletion occurs mainly in type I fibres with submaximal continuous exercise and in all three types, I, II A and II B, with intense exercise, performed intermittently or continuously. The glycogen saving effect in intermittent exercise is then not dependent on a specific fibre activation pattern.

The study was made possible by grants from the Swedish Medical Research Council (40X-04554).

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Acta physiol scand. 1978. 103. 456–462

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Contralateral influence on recruitment of curarized muscle fibres during maximal voluntary extension of the legs

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Abstract

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The force of maximal voluntary contraction (MVC) was compared during extension of one leg alone and during simultaneous extension of both legs. In 6 subjects MVC of two leg extension was 75±3.6 (S.E.) of the sum of the single one leg MVC. This may indicate a reduced muscle fibre involvement during two leg extension as compared with one leg extension. We investigated the possibility that either red (type I) or white (type II) muscle fibre recruitment was restricted during two leg extension. The neuromuscular transmission in the two types of fibres was partially blocked with d tubocurarine (dtc) or decamethonium (C_{10}) for either type I or II respectively In 5 subjects receiving dtc the ratio between the reduced two leg and one leg extension forces (75±2.3%) did not change During administration of C_{10}, however this ratio decreased by 16±3 1%. Partial blocking with dtc caused a two component curve during tension development with a first maximum at 0.22 s, and another maximum at 1.03 s of 3 s attempt to reach MVC. During partial blocking with C_{10} the first maximum disappeared indicating human fast twitch fibres to be blocked by C_{10}. Thus, partial blocking with C_{10} results in muscle contractions where a relatively large amount of type I muscle fibres are contributing to the force developed. The experiment suggests that type I fibre recruitment is restricted during severe static exercise in normal muscles. Furthermore it demonstrates that fibre recruitment can vary in the same muscle function performed with one leg or two legs.

Key words: d-tubocurarine decamethonium rate of rise of tension fast and slow muscle fibres motor unit recruitment; motor skill

The force of maximal voluntary contractions (MCV) is determined by the cross sectional area of the muscles and/or the ability of the nervous system for simultaneous tetanic recruitment of all synergistic motor units. Merton (1954) found the same force in the adductor pollicis muscle during MVC and electrical stimulation of the ulnar nerve. However in similar experiments Ikai *et al* (1967) saw a 30% increase in force during electrical stimulation. A study of Ikai and Steinhaus (1961) showed MVC to be influenced by a variety of external stimuli. In untrained subjects the verbal suggestion that strength was increased also increased MVC, but had no effect in a trained subject. Comparison of one and two leg MVC shows a lower two leg MVC than the sum of the two one leg MVC (Asmussen *et al* 1959 Asmussen and Heeboll Nielsen 1961 Secher *et al*. 1976) This is not the case in subjects (oarsmen) who perform repetitive two leg extension during training

(Secher 1975). These studies suggest that MVC only reflects full muscle strength in well trained subjects.

The present study was undertaken to investigate the possibility of a restricted red (type I) or white (type II) muscle fibre recruitment during simultaneous extension of the legs in subjects with low two to one leg MVC ratio. Partial neuromuscular block can be established for mainly type I or mainly type II muscle fibres. D-tubocurarine (dtc) is known to have a preference for neuromuscular transmission in red muscles while primarily white muscles are blocked by decamethonium (C_{10}) as shown in several animal species by Paton and Zaimis (1947). Subsequently Johansen *et al.* (1964) and Jorgensen *et al.* (1966) found a similar effect of dtc and C_{10} in human forearm and neck muscles. Thus, one and two leg MVC were compared during partial neuromuscular block with dtc and C_{10}. A preliminary note of the results has appeared (Secher *et al.* 1976).

Material and Methods

[illegible] male subjects (23–26 years), 5 of whom had extensive laboratory experience as subjects, participated in the study after their informed consent was obtained. Height and weight (mean ± S.E.) were 183 ± [illegible] cm and 80 ± [illegible] kg, respectively. The muscle groups tested were the leg extensors.

On the first day the force of MVC was recorded during 3 or 4 successive trials 30 to 60 [illegible] apart. A value was accepted as maximum when the following trial showed a decrease in force. Before and during the test the subjects were encouraged by the examiner to perform maximally. MVC during leg extensions was carried out on a strain gauge dynamometer according to Asmussen *et al.* (1959). During this test the subject is seated on an adjustable seat mounted in a horizontal iron frame. The feet are placed on a steel bar equipped with a plate at the center. The bar is mounted in bearings on the frame. The angle of the ankle joints is kept [illegible] 90° and the knee joint at 90°. Tests were performed as one and two leg MVC.

The strain gauges were connected to a Peekel instrument (581 DNH) and output read on an Elema-Schönander recorder at a speed of 2.5 cm [illegible] s^{-1}.

On the second day force development during MVC was first measured as described above and d-tubocurarine chloride (Abbott) 0.1 mg [illegible] kg^{-1} (3 subjects) or decamethonium bromide (Burroughs Wellcome) [illegible] to 0.015 mg [illegible] kg^{-1} (3 subjects) administered [illegible] through a catheter in the left forearm. A similar procedure was followed on the third experimental day on which occasion the alternative drug was used. The experimental days were chosen more than a week apart in order to avoid drug accumulation or water retention and muscle soreness from the use of C_{10}. During the action of the drugs recordings were made with 15 [illegible] intervals alternating between the right, left or both legs. An Ambu-E resuscitator apparatus was kept ready [illegible] all experiments, but there was in none of the experiments a need for it to be used.

Ordinary statistical methods were used to calculate means and standard error of the means (S.E.). [illegible] from the normal distribution was calculated and intra-individual differences were evaluated by Student's paired *t*-test (two tailed).

Results

Maximum force during MVC for the right and the left leg was 150 ± 22 and 130 ± 17 kp respectively (P [illegible] 0.6). For both legs it was 210 ± 23 kp or 75 ± 3.6 percent of the sum of the two one leg forces.

At the time of maximal action of the drugs the subjects had double vision and difficulty in maintaining head position, but all were ventilating adequately with no clinical sign of respiratory depression. Typical force recordings for a single subject are shown in Fig. 1. The maximum curves obtained are without dtc or C_{10}, respectively. Lower recordings were obtained during the action of the two drugs. Thus, the lowest recordings represented MVC during maximal action of the drugs. All curves were superimposed for direct comparison. At onset of the contraction a rapid and short lasting (easily fatiguable) component

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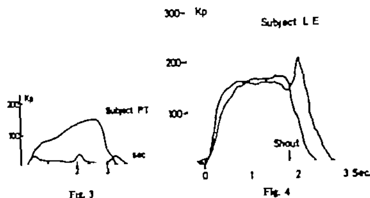


Fig. 3

Fig. 4

Fig. 3 Single recordings of force in uncurarized state and during administration of d-tubocurarine. Note that the fast component appears 3 times during an MVC recording

Fig. 4 Force development during MVC composed with and without shouting.

force maximum (control value 1.5 ± 0.18 s) tended to be lower during the partial neuromuscular blockings (for dtc expt. 1.3 ± 0.12 s and for C_{10} expt. 1.13 ± 0.08 s). The late component of the recordings appeared at a similar time after onset of the contraction during dtc and C_{10} expts. The early component had vanished at the time when the force of the non-blocked muscle reached the maximum of the late component.

The distributions of the times to the two force maxima for all recordings are shown in Fig. 2. Both the frequency distributions for the early and the late components were skewed to the right indicating an overrepresentation of slow contractions. Medians were (with 95 confidence limits) 0.22 (0.13–0.50) and 1.03 (0.64–2.7) s. Note that the early component only appeared in 411 of 807 recordings.

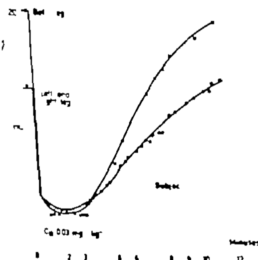


Fig. 5. Force maxima read from MVC recordings made before and after single injection of decamethonium.

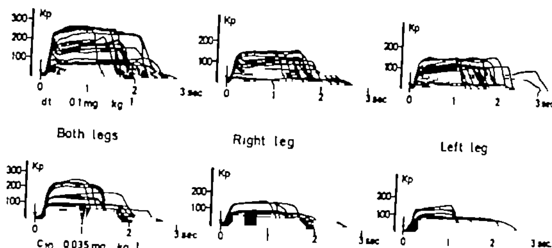


Fig. 1 Force recorded during MVC for one subject during extension right, left and both legs. *Upper tracing* d-tubocurarine expt. *Lower tracing* decamethonium expt. The curves for the expts. are superimposed.

appeared during blocking with dtc. This fast component was less frequent during the action of C_{10}. It was present in $39 \pm 5.9\%$ of the unblocked recordings and more often ($82 \pm 6.8\%$) in the dtc recordings than in the C_{10} recordings ($24 \pm 5.5\%$) ($P < 0.005$). The time to the early force maximum (control value 0.35 ± 0.06 s) was not significantly affected by administration of C_{10} (0.24 ± 0.03 s) or dtc (0.29 ± 0.03 s). The time to the late

Fig. 2. Frequency distribution of time to maximum for first and second component of force development.

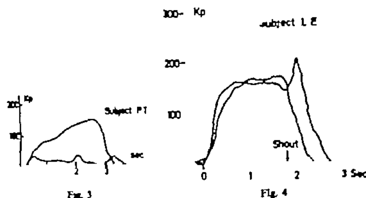


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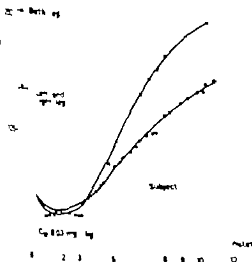


Fig. 5. Force maxima read from MVC recordings made before and after single i. injection of decamethonium.

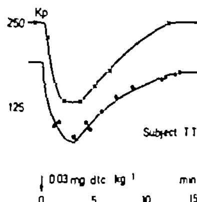


Fig. 6. Force maxima read from MVC recordings made before and after a single i.v injection of d-tubocurarine Signs as in Fig. 5

A fast component reappeared during the same contraction during renewed effort c the subject but force could not be maintained on this level and a marked fade in contrac tion was seen in each instance (see dtc recording of Fig. 3). Late appearance of the fa component could also be elicited by exceptional stimulation such as a shout—cf th recording in the unblocked state in Fig. 4

The variation in maximum force reached at repeated contractions during drug admini tration is illustrated in Fig. 5 and 6 for C and dtc, respectively In the 5 experience subjects similar smooth curves could be drawn using approximately all force determination as estimates of MVC. But in the inexperienced subject this was not possible, and his dat were therefore not used in this part of the study Occasionally recovery MVC exceede MVC recorded before drug administration (Fig. 5, one leg values)

Ratios between two and one leg extension forces were similar before and after recover from drug administration (Table I) The average was used as control value. These ratio were reproducible from day to day However two leg maximum force was relatively mor reduced during administration of C than one leg maximum force. dtc reduced one an two leg maximum forces to approximately the same extent. Two examples with particularl large falls in force development are shown in Fig. 5 and 6. During maximum effect of C

TABLE I Ratios between MVC during extension of both legs and MVC of right plus left leg.

		no.	dtc	C_{10}	Difference	±S.E.	t value
Before medication	mean	5	77	81	−4	1 4	2.66
	±S.E.		.9	3 6			
After medication	mean	5	74	80	−6	5.2	1 12
	±S.E.		2 2	4.5			
Difference		5	3	1			
	±S.E.		2.1	4.0			
t value			1 41	0.25			
Average of before and after" values	mean	5	75	80	−5	3.3	1 48
	±S.E.		2.3	3.5			
During maximal ac tion of the drugs	mean	5	77	64	13	2.6	5 1
	±S.E.		3.8	4.0			
Difference	mean	5	–	16			
	±S.E.		5.7	3 1			
t value			0.37	5 19			

two leg force was equal to one leg force (Fig. 5). During the maximum action of dtc the two leg/one leg force ratio tended to be higher than that observed in the unblocked state (cf. Fig. 6).

Discussion

Contraction pattern

The times to maximum force observed here for the fast and slow components of force development are in the same order of magnitude as that noted during tetani of fast and slow rat motor units (Stieg 1964, Close 1967). In the present study the distributions of the times to maximum force were skewed towards the right indicating an overrepresentation of slow elements within both components. If the subjects used an effort less than maximal at onset of contraction (e.g. the uncurarized MVC recording of Fig. 3, this will tend to delay the time to maximum force and thereby obscure the two component pattern. In general, the force pattern recorded by us during blocking supports the concept, that a fast and slow type of human muscle fibres work together during voluntary contraction. It also confirms earlier findings of the specificity of a blocking by dtc and C_{10} of human type I and II muscle fibres, respectively (Mofbech and Johansen 1969, Bonde-Petersen *et al.* 1975).

The fast component initiates the contractions recorded. The data of Grimby and Hannerz (1968) suggested a change in the recruitment pattern in human skeletal muscle only during contraction of high intensity (i.e. high velocity). The present data suggest that a gradual change in muscle fibre recruitment takes place during intensive muscular contractions because fatigue in the fast contracting fibres appears rapidly. However the MVC recordings during C_{10} blocking show that slow fibres also can be active at onset of contraction. The fast contracting element only reappears with renewed effort. This may explain the increase in the recorded strength after a shot or a shout as described by Ikai and Steinhaus (1961).

Thorstensson *et al.* (1976) saw a significant correlation between force and type II muscle fibre area only at high contraction velocities. The present recordings support the finding that the fast contracting fibres are of importance for the rate of rise of tension rather than for the absolute force reached.

Muscle fibre recruitment

The present data (Table I, Fig. 5 & 6) indicate a restricted type I muscle fibre recruitment during simultaneous two leg extension.

Asmussen *et al.* (1959), Asmussen and Heebøll-Nielsen (1961) and Secher *et al.* (1976) showed, as confirmed in the present study, two leg extension force to be less than the summated one leg extension force except in subjects specially trained for two leg extension (e.g. oarsmen, Secher 1975). These studies indicate that muscular performance during complex motor activity can be improved by training specifically for one or two leg achievements.

According to Henneman (1957) and Henneman and Olson (1965) it would be expected that type I muscle fibres contained in small motor units with a low activation threshold are active whenever type II fibres are recruited. The present results indicate a restricted type I muscle fibre recruitment during two-legged MVC. However the work of Henneman

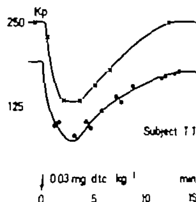


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Average of "before and after" values	mean	5	75	80	−5	3.3	1.48
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t value			0.37	5.19			

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group was performed in decerebrated cats. At maximal effort cortex cerebri might modify the function of the motor neurons. It was noted by Agnew and Preston (1965) that cortical stimulation of a pyramidal preparation resulted in either facilitation or inhibition of some motor neurons within a population innervating the same muscle, while both inhibitory and facilitatory influence was seen in others. It is in line with the present results that cortical stimulation in a similar preparation (Preston and Whitlock 1963) facilitates monosynaptic reflexes over the "white" gastrocnemius, but inhibits reflexes over the "red" soleus muscle. Thus, the neurologic component of the training response conforms to a decrease in cortical inhibition of type I motor units.

This study was made possible by grants from Frk Brandts and Kobmand I Odense Johann og Hanne Weimann s foundations.

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Acta physiol. scand. 1978. 103. 463–471

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A study of permanent adipsia induced by medial forebrain lesions

By

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Received 9 February 1978

Abstract

RUNDGREN, M. and F. FYHRQUIST. *A study of permanent adipsia induced by medial forebrain lesions.* Acta physiol. scand. 1978. *103.* 463–471.

Cerebral lesions involving most of the anterior wall of the third ventricle, and the medial part of the septal region, induced a permanent loss of thirst in two goats. The ventral part of the lamina terminalis remained intact in one of the animals. Pronounced dehydration (10–13 % loss of b.wt.) developed during periods of 6–7 days when water supplementation was omitted. Determinations of plasma arginine vasopressin in one of the animals revealed that the dehydration did not cause any significant increase in the secretion of antidiuretic hormone. However, the water deficit induced a considerable rise in plasma renin activity and tachycardia. If anything, the carotid blood pressure became slightly elevated towards the end of 7 d dehydration periods. The lesions obviously inactivated a cerebral sensory mechanism controlling water balance. It may have been due mainly to destruction of juxtaventricular receptors in the anterior hypothalamic region, but perhaps also to a disruption of afferents from such receptors located posterior to this lesioned level.

Previous ablation studies have revealed that structures in, or close to the frontal wall of the third cerebral ventricle are essential for the maintenance of body fluid homeostasis (Andersson, Leksell and Lishajko 1975, Buggy and Johnson 1977). Medially placed forebrain lesions which involved the entire anterior wall of the third ventricle were observed to cause a complete and persistent absence of the urge to drink in the goat (Andersson *et al.* 1975). The regulation of antidiuretic hormone (ADH) secretion was apparently also impaired in the adipsic animals, since very little antidiuretic activity could be detected in the urine when the goats were severely dehydrated. With the use of a sensitive radioimmunoassay method (Fyhrquist, Wallenius and Hollemans 1976 b) it has recently been shown that moderate dehydration, leading to a 5 % elevation of plasma [Na$^+$], causes roughly a 5-fold increase in

the plasma ADH concentration of normal goats (Olsson *et al* 1978) This made it of interest to study with the same method, the ADH release in response to dehydration in the goat made adipsic by medial forebrain damage. Since activation of the renin-angiotensin system may play a role in the regulation of the ADH-secretion (*cf* Severs and Daniel-Severs 1973) it was also of interest to make parallel determinations of plasma renin activity (PRA). A further aim of the present study was to obtain if possible, more information about the extent of forebrain damage necessary to induce adipsia.

Methods

Animals Two female goats (in the following named I and II) were used The animals (pre-lesioning b. t. 32 and 28 kg) were kept in metabolism cages (separating urine from feces) at room temperature (20±1°C), where they had free access to hay and water To maintain the goats in positive sodium balance they were each afternoon given 6 g of NaCl dissolved in 200 ml of tepid water During the entire observation period both goats drank this salt solution voluntarily The 24 h water intake and urine output were measured round 8 a.m. when samples of urine were taken for analyses.

Electrode implantation and lesioning technique Both animals had a pair of thermocouple electrodes (o.d. 0.7 mm) permanently implanted into the preoptic/anterior hypothalamic region. The implantations were performed under general anesthesia (nembutal) 12 (Goat I) and 4 (Goat II) weeks before the electrodes were used for production of radio-frequency (RF) lesions. Goat I had the electrodes placed along the midline of the brain with an interspace of 4 mm, and the other animal had the electrodes bilaterally implanted 3 mm apart. The length of the uninsulated ends of the electrodes was 5 and 3 mm respectively Coagulative lesions were made by applying RF energy between the thermocouple electrodes as previously described in detail (Andersson *et al* 1975).

Water supplementation and hydration Since the brain damage induced adipsia, a daily water supplementation was administered by stomach tube to the rumen during the post-lesioning observation period. The amount of water (38°C) was adjusted to maintain roughly normal body weight and plasma [Na]. The water supplementation was omitted during dehydration tests. On some occasions an additional load of water (75 or 150 ml/kg b wt.) was given in order to test the animal' ability to eliminate excessive body water

Routine sampling and analyses. To obtain clean urine samples during dehydration and hydration experiments, urine was collected via a Foley catheter Jugular vein blood samples (10 ml) were drawn into heparinized syringes. For determination of the hematocrit (Ht) the blood samples were centrifuged in graded tubes at 3 000 rpm for exactly 7 min. Urine and plasma [Na] and [K] were measured by use of an "IL 343" internal standard flame photometer An Advanced Instruments Inc. osmometer was used for determination of plasma and urine osmolality

Hormone analyses. Ice-chilled syringes with 0.3 M EDTA as anticoagulant were used to obtain blood samples for radioimmunoassays. The blood was immediately centrifuged at +4°C, and the plasma was separated and stored at −20°C until radioimmunoassayed. Determinations of plasma arginine vasopression (AVP) were performed with a method previously described (Fyhrquist *et al.* 1976 b). The sensitivity of this method is 0.2 pg/ml. Radioimmunoassays of plasma renin activity (PRA) were made according to Fyhrquist *et al* (1976 a).

Blood pressure and heart rate recordings Both animals had a polyvinyl catheter permanently implanted via the superficial temporal artery into the carotid artery as described by Eriksson, Fernández and Olsson (1971). The systolic/diastolic and the mean carotid blood pressures were recorded on an ink-writing polygraph via a Statham pressure transducer When not registered during blood pressure recording, the heart rate was determined at intervals by auscultation.

Histology At the end of the post lesioning observation period the animals were decapitated under nembutal anesthesia. The heads were perfused with isotonic saline followed by formalin/saline. After formalin fixation, a block of the brain (including the preoptic region, the hypothalamus and the thalamus) was embedded in celloidine and cut at 30 μ by serial transverse (Goat I), or sagittal (Goat II) sectioning. The sections were stained either with toluidine blue, or according to Loyez (*cf* Culling 1957) with neutral red as counter-staining.

Figures followed by ± represent mean ± S.E. Student's t test was used for statistical evaluations.

Results

I. *Pre-lesioning studies*

Balance studies

During the period between electrode implantation and lesioning the daily water intake remained rather stable in both goats (40–45 ml/kg). The urine output was 60–70% of the water intake and the urine osmolality varied between 700 and 1 700 mosm/kg. Great variations in renal excretion of Na (25–250 mmol/day) and K (60–230 mmol/day) were seen. Blood samples taken repeatedly during the pre-lesioning observation periods revealed that the blood plasma composition remained within the range earlier found in water replete normal goats ([Na] 151.4 ± 0.6 mmol/l, [K] 4.2 ± 0.1 mmol/l, osmolality 295 ± 2 mosm/kg) (Rundgren and Fyhrquist 1978). The Ht was within the range 29 to 36. Pre-lesioning plasma AVP and PRA were only determined once in Goat II. The values obtained were: AVP 3.1 pg/ml and PRA 1.19 ng/ml h^{-1}.

Thermal and electrical stimulation

During the pre-lesioning observation periods the animals were repeatedly subjected to either moderate local forebrain warming (Goat I), or to bipolar electrical stimulation (Goat II) via the permanently implanted thermocouple electrodes. In Goat I the forebrain heating induced delayed drinking, and post-stimulatory hyperdipsia was observed in Goat II. These experiments have been described in detail previously (Rundgren 1978).

II. *Post-lesioning studies*

Acute effects

Both animals temporarily reacted with polypneic panting to the RF coagulation of forebrain tissue. Goat I also showed 5-fold increase in water intake during the first post-lesioning 24 h period. No water diuresis developed in response to this over-hydration which caused considerable hemodilution (fall in plasma [Na] to 132 mmol/l and plasma osmolality to 262 mosm/kg). No acute dipsogenic effect was observed in Goat II, which became adipsic immediately upon lesioning.

Chronic effects

Adipsia. Apart from the acute over-drinking seen in Goat I, none of the goats drank water spontaneously during the 90 and 93 days they were observed after lesioning. However both animals always eagerly drank the 200 ml of 3% NaCl solution they were offered daily as extra salt supply. On five (Goat I) and seven (Goat II) occasions the regular water supplementation was interrupted for 3 to 7 days. For ethical reasons, these dehydration periods were always terminated when the goats showed a tendency to reduce their food intake, i.e. when the water deficit had caused 10 to 13% reduction of the body weight. Peak plasma values at this degree of dehydration were [Na] 183 mmol/l, [K] 5.5 mmol/l and osmolality 395 mosm/kg, and the Ht had increased by about 40%. Apart from the complete lack of an urge to drink water and the slight reduction of appetite, the goats behaved normally when dehydrated to this extent.

the plasma ADH concentration of normal goats (Olsson *et al* 1978). This made it of interest to study with the same method the ADH release in response to dehydration in the goat made adipsic by medial forebrain damage. Since activation of the renin-angiotensin system may play a role in the regulation of the ADH-secretion (*cf* Severs and Daniel-Severs 1973) it was also of interest to make parallel determinations of plasma renin activity (PRA). A further aim of the present study was to obtain, if possible, more information about the extent of forebrain damage necessary to induce adipsia.

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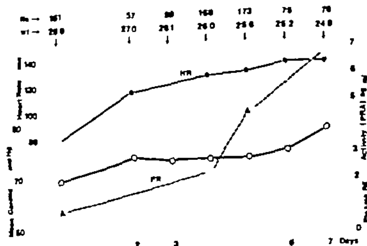


Fig. 2. Hemodynamic events and PRA during period when water supplementation was omitted in Goat II. Note the steep rise in PRA starting when the progressive water loss had reduced 7 reduction of body weight. Plasma [Na+] expressed in mmol/l. B.P. = Blood pressure. B.wt. = Body weight in kg. b/min = beats min.

at a plasma [Na] of 164 mmol/l. An appropriate reduction of renal water loss occurred during the dehydration periods, in spite of the fact that (according to the data obtained in Goat II) the ADH-secretion was not significantly increased. Thus, urine flow became reduced to roughly 0.1 ml/min with a concomitant rise in urinary osmolality to a level between 2000 and 2 500 mosm/kg. This dehydration-induced reduction in urine flow was accompanied by a relatively low renal Na excretion (10 to 50 μmol/min).

The inability of Goat I to eliminate by a water diuresis the large amount of water consumed during the first 24 post-lesioning hours (see above) suggests that the forebrain lesioning induced an uncontrolled release of ADH. Also during later post-lesioning hydration tests the animal exhibited impaired ability to excrete excessive water. Thus, positive renal free water clearance was not seen until the water load had induced abnormal hemodilution (plasma [Na] 135 mmol/l). As reported previously (Rundgren and Fyhrquist 1978) the presence of post-lesioning inappropriate ADH-secretion was even more obvious in Goat II, and as in that animal confirmed by plasma AVP determinations during hydration.

Hemodynamics and PRA. The fact that both goats had completely lost the urge to drink water provided the possibility to study hemodynamic reactions to developing dehydration without the stressful interference of unsatisfied thirst. Fig. 2 illustrates the observed hemodynamic events, and PRA changes during a 7 d dehydration period in Goat II. As seen in this figure, the progressive hypovolemia developing during the dehydration periods was accompanied by an increase in heart rate. The tachycardia reached a plateau round 140 beats/min when the water deficit had induced about 10% reduction in body weight. The degree of dehydration induced in the present experiments (10 to 13% decrease in b.wt.) was in no case observed to cause any fall in the mean carotid blood pressure. Instead, slight increase in blood pressure was observed towards the end of the dehydration periods. At the same time PRA reached very high levels (Fig. 1 and 2). Therefore, pronounced activa-

TABLE I

	Pre-dehydration (n = 8)	Moderate dehydration (n = 8)	Pronounced dehydration (n = 10)
Plasma [Na] (mmol/l)	150.0 ± 0.8	155.0–164.9	165.0 – 175.0
Plasma osmolality (mosm/kg)	295.5 ± 3	303–343	342 376
Plasma AVP (pg/ml)	3.90 ± 0.68	3.35 ± 0.39	5.17 ± 1.01[c]
PRA (ng/ml h⁻¹)	1.27 ± 0.29	2.44 ± 0.30[b]	3.73 ± 0.48[c]

Statistical significance from pre-dehydration values. N.S. [b] $p < 0.02$. $p < 0.001$
Post lesioning plasma arginine asopressin (AVP) and plasma renin activity (PRA) in Goat II during normal water balance, and at different degrees of water deficit during 6 dehydration periods.

ADH secretion. Determinations of plasma AVP were made in Goat II when the animal was supplemented with water to pre-lesioning body weight and plasma [Na], and during 6 out of 7 dehydration periods The values obtained at plasma [Na] up to 175 mmol/l are presented in Table I The AVP was not elevated during moderate dehydration (3 to 10% increase in plasma [Na]), whereas a slight, statistically insignificant increase was observed during more pronounced dehydration (10 to 17% rise in plasma [Na]). At the end of one of the dehydration periods, AVP was determined when the plasma [Na] was as high as 182 mmol/l A value of 7 7 pg/ml, *i.e* twice mean pre-dehydration AVP concentration was observed. However on rehydration (Fig. 1) AVP returned to pre-dehydration level already

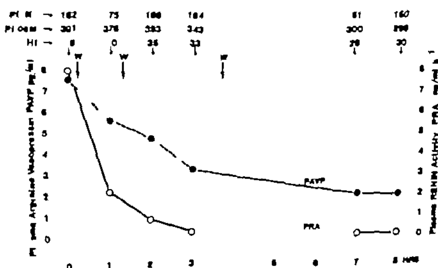


Fig. 1 The effect of gradual rehydration on plasma AVP and PRA in Goat II after 7 day period of omitted water supplementation which had induced pronounced dehydration. The elevation of AVP seen at the end of the dehydration period is only a fraction of that induced in the normal goat by 2 days of water deprivation (Olsson *et al.* 1978). Note that AVP returns to pre-dehydration level (see Table I) when plasma [Na⁺] and osmolality (= figures on top) remains considerably elevated above normal. *W* = Administration of 38°C water by stomach tube (100, 70 and 70 ml/kg respectively). Plasma [Na] and osmolality on top of the figure are expressed in mmol/l and mosm/kg. Ht = hematocrit.



Fig. 4. A slightly oblique midsagittal section (30 μ) showing the extent of coagulative brain damage in Goat II (delineated by broken line). Arrow marked *L* indicates the anterior border of the lesion. *E* = electrode track; 3 = Third ventricle; CC = Corpus callosum; CM = Corpus mammillare; CO = Chiasma opticum; HYP = Anterior lobe of the hypophysis; MI = Massa intermedia; OV = Organon vasculosum of the lamina terminalis. Stain: Loyez. Counter staining with neutral red explains the darkness of the anterior and the intermediate lobes of the hypophysis.

reflex, tonic inhibitory influence upon the release of ADH (*cf.* Andersson 1977). However substantial increase in ADH-secretion as effect of hypovolemia does apparently not occur until the blood volume is reduced to such an extent that the arterial blood pressure falls (Dunn *et al.* 1973; Arnauld *et al.* 1977).

Among the supports for the idea that juxtaventricular receptors in the anterior hypothalamic region play an important role in the regulation of water intake and ADH-secretion are the results of ablation studies in the goat (Andersson *et al.* 1975) and the rat (Buggy and Johnson 1977). In both species, medially placed lesions destroying the whole anterior border of the third ventricle have been found to induce adipsia. To judge from bioassays of urinary antidiuretic activity in the goat, such lesions also impair ADH-secretion in response to dehydration. The latter observation has been confirmed and extended in the present study. Determinations of plasma AVP made in Goat II before, and at intervals after water supplementation had been omitted, revealed that a water deficit causing >10% increase in plasma [Na] did not cause any significant increase in the ADH-secretion (Table I), although the cerebral damage in this goat (Fig. 3 and 4) left the supraoptic and paraventricular nuclei intact. However, a conspicuous rise in PRA was seen at this degree of dehydration. Since in normal goats dehydration to less than half that extent causes large increases in plasma AVP (Olsson *et al.* 1978), it suggests that the forebrain lesion in Goat II severely impaired a regulation of ADH-secretion which is dependent upon extracellular [Na] and, eventually upon activation of the renin-angiotensin system. The relatively small (about two-fold) increase in AVP observed during a water deficit elevating plasma [Na] to 182 mmol/l (Fig. 1) indicates that some residual regulation dependent upon extracellular [Na] and/or

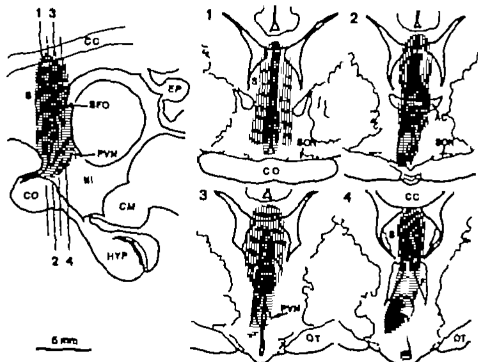


Fig. 3 A midsagittal section through the hypothalamic region of the goat (left), and four transverse sections (corresponding to the numbered levels on the sagittal section). Cerebral damage induced in Goat I *horizontally hatched* in Goat II – *vertically hatched* III – Third ventricle AC – Anterior commissure CC – Corpus callosum CM – Corpus mammillare CO – Chiasma opticum EP – Epiphysis, F – Descending column of the fornix, HYP – Anterior lobe of the hypophysis, OT – Optic tract; PVN – Paraventricular nucleus, S – Septal region SFO – Subfornical organ SON – Supraoptic nucleus.

tion of the renin-angiotensin system may have contributed to preserve, or even increase the blood pressure On rehydration, the PRA rapidly returned to pre-dehydration, or even lower levels (Fig. 1).

Forebrain lesions The extent of the cerebral lesions in Goat I and II is projected on midsagittal and transverse sections in Fig. 3 (horizontally and vertically hatched areas, respectively) The brain damage in common (= crosshatched areas) destroyed most of the anterior wall of the third ventricle and the medial portion of the septal region. However a fraction of the anterio-ventral border of the third ventricle (including most of the organum vasculosum of the lamina terminalis, and the supraoptic recess) was untouched by the lesion in Goat II (Fig. 4).

Discussion

Much experimental evidence favours the new concept that thirst and ADH-secretion in response to elevated extracellular [Na] and osmolality are mediated by sodium sensitive receptors which predominantly are located close to the anterior wall of the third ventricle. The same cerebral sensory mechanism appears to become engaged also when hypovolemia-induced activation of the renin-angiotensin system elicits thirst and ADH release. In addition, impulses from cardiovascular distention receptors and baroreceptors seem to exert a

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angiotensin II might have been present. However, this elevation of AVP could also have been due to diminished cardiovascular inhibition of the ADH-secretion.

An uncompensated transient water diuresis, indicating a sudden elevation of the stimulus threshold for ADH release and thirst, has previously been observed as an acute effect of medially placed forebrain lesions (Andersson *et al.* 1975), and lesions mainly confined to the septal region (Rundgren and Fyhrquist 1978). No such acute effect of lesioning was obtained in the present animals. The reason by all probability is that, in addition to adipsia and elevated dehydration threshold for ADH-release, the cerebral damage caused apparent inappropriate ADH-secretion. As discussed in a previous paper (Rundgren and Fyhrquist 1978), the latter effect may have been due to the fact that the lesions on one, or both sides, extended near the supraoptic nuclei (Fig. 3, transverse sections 1 and 3), and hereby interrupted pathways mediating reflex volumetric inhibition of the ADH release.

Although a persistent effect of the present forebrain damage was adipsia, one of the animals (Goat I) paradoxically exhibited transient hyperdipsia as acute effect of lesioning. A near at hand explanation is that lesioning in this animal temporarily caused irritative stimulation of "thirst-pathways" at the border of the region affected by the RF-heating. A non-specific stimulation of this kind might have occurred in the septal region, since here the permanent brain damage was much more narrow than in Goat II, which did not drink at all after lesioning (Fig. 3, transverse section 1).

The ventral limitation of the cerebral damage in Goat II demonstrates that adipsia in fact can be induced by forebrain lesions leaving most of the organum vasculosum of the lamina terminalis intact. This has not become evident from previous ablation studies. It does not exclude the possibility that receptors involved in the control of water balance are located also in the most anterio-ventral fraction of the ventricular wall, since the lesion might have interrupted pathways mediating receptor information from that area. Furthermore, the present study by no means disproves that juxtaventricular receptors regulating water intake and ADH-release also may be distributed posterior to the frontal border of the third ventricle. Afferents from receptors of that location could well have been disrupted by the forebrain lesions induced here. Of some interest with regard to this possibility is that infusions of hypertonic NaCl and angiotensin II into the fourth ventricle recently have been observed to cause apparent release of ADH in hydrated goats (L. G. Leksell and M. Rundgren, unpublished).

This work was supported by grants from Karolinska Institutet, from the Swedish Medical Research Council (Project 04X-00503), and from the Finnish National Research Council for Medical Science (Project 2224/3).

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Acta physiol. scand 1978 103, 472–474
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Effects of hypoxia on the rat portal vein *in vitro* P_O gradients in tissue and surrounding fluid

By

PER HELLSTRAND

Myogenic spontaneous activity of the isolated rat portal vein decreases with lowered P_{O_2} of the bath medium (Hellstrand, Johansson and Norberg 1977) Graded inhibition occurs at $P_{O_2} < 50$ mmHg and is caused by depression both of electrical membrane activity and of energy supply to contraction An anoxic core in the muscle at ambient P_{O_2} as high as 30–50 mmHg was not considered likely on estimation of the P_{O_2} drop within the muscle wall Consequently it was suggested that hypoxia might influence the smooth muscle cells themselves in a graded way over a limited P_{O_2} range.

Recently Fay Nair and Whalen (1977), using micro-P_{O_2}-electrodes, have demonstrated a significant P_{O_2} drop near the surface of a guinea pig ductus arteriosus mounted in vitro, presumably due to an unstirred layer of fluid surrounding the preparation The possibility arises that such an effect, leading to an anoxic core in the vessel wall, might explain the graded response of the portal vein to hypoxic solutions. In the present study this problem has been approached by mounting a conventional membrane-covered polarographic P_{O_2} electrode in direct contact with the surface of portal veins to get an estimate of the minimal P_{O_2} to which the smooth muscle cells are exposed Simultaneously some observations could be made on the temporal relation between phasic contractions and oxygen consumption.

Portal veins were cut open longitudinally spread out as rectangular plates (approx. 0 1 3–4 × 6–9 mm), and mounted horizontally between a micrometer and a Grass FT03 force transducer An O_2 electrode located vertically above the strip could be lowered to make contact with its surface. The muscle and electrode were mounted in a 50 ml organ bath at 37°C filled with bubbled Krebs solution After equilibration for about 45 min P_{O_2} was lowered in steps as previously described (Hellstrand *et al* 1977) The polarographic O_2 electrode (Radiometer Copenhagen, Denmark) had its tip shaped as a flat disc, large enough to cover the whole muscle sheet. Some experiments were run using an electrode with a half spherical tip (L. Eschweiler & Co Kiel, Germany) providing a smaller contact area with the muscle. No difference was apparent in the results obtained with the two electrodes.

Fig. 1 shows isometric spontaneous activity of a portal vein, its time integral and the O_2-electrode recording. At first the electrode tip is located in the bath fluid 5 mm above the strip. As it is lowered to touch the muscle (first arrow) P_{O_2} falls from 115 mmHg to around

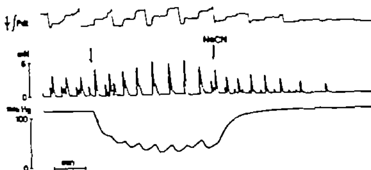


Fig. 1. Spontaneous activity of portal vein (middle trace), time integral of tension over 1 min periods, in arbitrary units (upper trace), and O_2 electrode recording (lower trace). P_{O_2} in bath medium 115 mmHg. First arrow: electrode lowered 5 mm to make contact with muscle. Second arrow: addition of 2 mM NaCN.

33 mmHg. It is seen that this surface P_{O_2} oscillates in pace with the spontaneous contractions. At the second arrow respiration of the muscle is blocked by addition of 2 mM NaCN. With the electrode still lowered, P_{O_2} returns to 115 mmHg. Thus, O_2 consumption by the electrode does not appreciably influence P_{O_2} recorded at the muscle surface. Even though contractions persist for a few minutes in the poisoned muscle there are no oscillations on the rising P_{O_2} curve (cf. the falling curve). This indicates that those seen in the unpoisoned muscle are not movement artifacts but signs of varying O_2 uptake. Neglecting electrode delay it was a consistent finding that surface P_{O_2} started to fall near the peak of contraction. Apparently the cell respiration responds very quickly to changes in contractile activity.

In Fig. 2 the integrated spontaneous activity recorded with the electrode lowered is plotted vs. solution P_{O_2} (filled circles) and vs. surface P_{O_2} (open circles), respectively (6 expts). At each P_{O_2} level spontaneous activity was integrated for 6 min. Solution P_{O_2} was recorded immediately before and after this period by raising the electrode 0.4–5 mm. In

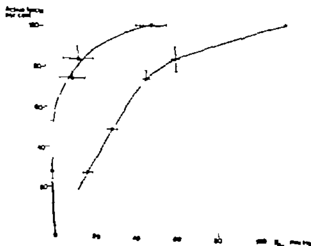


Fig. 2. Integrated spontaneous activity vs. P_{O_2} in bath (solid circles) and vs. P_{O_2} at muscle surface (open circles). 6 experiments. Means ± SE shown. Curves drawn by eye. Integrated activity at control P_{O_2} (115 mmHg) set at 100 %. All points refer to mechanical activity measured with electrode at muscle surface.

this range, solution P_{O_2} was independent of distance from the muscle. It is seen from the open circles that a dramatic reduction of activity occurs only when surface P_{O_2} is very low 0–2 mmHg. It must be remembered that in these experiments one surface only of the strip is exposed to bubbled solution By symmetry then, the electrode can be thought of as measuring P_{O_2} in the centre of a hypothetical strip of double thickness (*i.e* about 0.2 mm) oxygenated at both surfaces. The O_2 consumption of the portal vein is about 0.55 µmol/min g (Hellstrand 1977). The P_{O_2} drop from surface to centre of a 0.2 mm muscle strip can thus be estimated at 36 mmHg (*cf* Pittman and Duling 1973). At control P_{O_2} in the bath, a difference of 60–70 mmHg between solution and surface P_{O_2} was observed (Fig. 1 and 2) Roughly half of this may thus be expected to be located outside the vessel wall, indicating the presence of an unstirred layer of about 100 µm in the solution close to the muscle (R. N Pittman, personal communication). Assuming such a layer to exist also with no electrode present the P_{O_2} drop in the solution near the surfaces of a 0 1 mm strip can be calculated to 10–15 mmHg. With the above assumptions, the P_{O_2} drop within the strip is about 9 mmHg. The observed range of 40–50 mmHg for an effect of P_{O_2} on the muscle activity thus corresponds to 20–25 mmHg at the level of the innermost cells. Still, many estimates of "critical tissue P_{O_2}" indicate a value of only a few mmHg (Pittman and Duling 1973 Fay *et al* 1977) A possible explanation for the difference might be, first, the oscillatory behaviour of the O consumption noted in this study leading to a "part-time anoxia for some of the cells, and, second the effects of hypoxia on the electrophysiology of the portal vein. Since this smooth muscle carries propagated action potentials, hypoxic depression of a pacemaker region will influence other parts of the preparation. These electrophysiological effects of hypoxia, are presently under study

Valuable suggestions concerning effects of unstirred layers in vitro and the means to study them have been given by Drs B. R. Duling and R. N. Pittman. Miss M Lundahl, M Heidenholm and I. Nordström have given able technical assistance. The study was supported by the Swedish Medical Research Council (grant 04X-00028), the Medical Faculty University of Lund, and AB Hässle, Göteborg.

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Acta physiol. scand. 1978. 103. 475–477

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Enkephalin-like immunoreactivity in nerve terminals in sympathetic ganglia and adrenal medulla and in adrenal medullary gland cells

By

Marianne Schultzberg, Tomas Hökfelt, Jan M. Lundberg, Lars Terenius, Lars-Gösta Elfvin and Robert Elde

Recently Hughes *et al.* (1975) have isolated and structurally identified two pentapeptides from the brain, the enkephalins, which may represent endogenous ligands for the opioid receptors (Terenius and Wahlström 1974, 1975 Hughes *et al.* 1975, Pasternak *et al.* 1975). Their distribution has been studied with biochemical and histochemical techniques both in the central (Simantov *et al.* 1976, Smith *et al.* 1976, Hong *et al.* 1977 Hughes *et al.* 1977 Elde *et al.* 1976, Hökfelt *et al.* 1977 a, Simantov *et al.* 1977 Watson *et al.* 1977) and peripheral (Elde *et al.* 1976, Hughes *et al.* 1977) nervous system. In the present paper preliminary results on the distribution of enkephalin-like immunoreactivity in sympathetic ganglia and the adrenal gland of guinea pigs are reported.

Male guinea pigs (b.wt. 300–400 g) were anesthetized and perfused with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer solution. After 30 min the inferior mesenteric ganglion and the adrenal gland were dissected out, immersed in the same fixative for at least 90 min, rinsed in sucrose containing buffer cut on a cryostat and processed for immunocytochemistry according to Coons and collaborators (see Coons 1958). The sections were incubated in a humid atmosphere for 24 h at +4°C with antiserum raised to methionine enkephalin (met ENK) conjugated with bovine serum albumin (BSA). The serum had been pretreated with BSA and a dilution of 1 160 was used. After rinsing in phosphate buffered saline (PBS), second incubation was performed at +37°C for 30 min with fluorescein-isothiocyanate (FITC) conjugated antibodies (Statens Bakteriologiska Laboratorium, Stockholm, Sweden). The sections were mounted in glycerine-PBS (3 1) and examined in a Zeiss fluorescence microscope. Met-ENK antiserum pretreated with an excess of met ENK (100 µg/ml antiserum diluted 1 160) served as a control serum.

In the *inferior mesenteric ganglion* a very dense plexus of ENK-immunoreactive, varicose nerve terminals was observed. Strands of several terminals surrounded the majority of the principal ganglion cells (Fig. 1). Occasionally small groups of principal ganglion cell profiles were seen which in single sections did not seem directly related to fluorescent fibers. No principal ganglion cells, but few small intensely fluorescent (SIF) cells (see Eränkö 1976) were met ENK positive. No fluorescent fibers were observed around blood vessels.

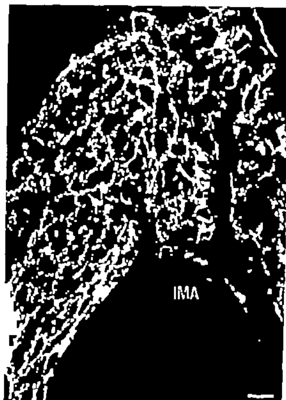


Fig. 1



Fig. 2

Fig. 1 Immunofluorescence micrograph of the inferior mesenteric ganglion after incubation with met ENK antiserum. A dense plexus of immunoreactive nerve terminals is seen around the principal ganglion cells. IMA = inferior mesenteric artery. Bar indicates 50 μm.

Fig. 2 Immunofluorescence micrograph of the adrenal gland after incubation with met ENK antiserum. Strongly fluorescent gland cells and some fluorescent nerve terminals (arrows) are seen. Asterisks indicate adrenal cortex. Bar indicates 50 μm.

In the *adrenal medulla* a large number of gland cells exhibited met ENK immunoreactivity of varying intensity. Among the fluorescent gland cells a sparse network of varicose met-ENK positive nerve terminals could be seen (Fig. 2).

The present findings suggest that nerve terminals in some sympathetic ganglia and in the adrenal gland contain an enkephalin-like peptide. Other biologically active peptides, such as substance P and VIP have also been found here (Hökfelt *et al* 1977 b, c). Thus, the peripheral autonomic nervous system may contain a further class of neurons, peptide neurons, in addition to classical cholinergic and adrenergic systems. Besides nerve terminals, a large proportion of gland cells of the adrenal medulla seems to contain the same or a similar peptide. This is another example of the presence of a biologically active peptide in amine containing cells which is characteristic of so-called APUD (Amine content and/or amine Precursor Uptake and Decarboxylation) cells (Pearse 1969).

This study was supported by grants from the Karolinska Institutet, the Swedish Medical Research Council (04X 2887, 12X 5189, 25X 5065), Magnus Bergvalls Stiftelse, Harald Jeanssons Stiftelse and Harald and Greta Jeanssons Stiftelse.

The skilful technical assistance of Miss Gun Norell and Mrs Waldtraut Hiort is gratefully acknowledged.

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Acta physiol. scand. 1978. 103 478–480

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Failure of somatostatin to eliminate the glucagon release induced by baroreceptor unloading in the cat

By

JOHANNES JÄRHULT BO AHRÉN, MATS ERICSSON and JENS HOLST

Several studies have demonstrated that somatostatin can suppress the release of pancreati glucagon both in animals and man. Thus, an infusion of somatostatin lowers the basa glucagon level (see Luft *et al* 1978) and somatostatin almost totally blocks the stimulator effect on the glucagon release induced by insulin hypoglycemia (Chideckel *et al* 1975) arginine (Gerich *et al* 1974), adrenaline (Weir *et al* 1974), orciprenaline (Raptis *et al* 1977) or isoproterenol (Gerich *et al* 1975) It has been shown recently by our group that large amounts of glucagon are reflexly released into the portal circulation in response to unloading of the carotid baroreceptors in the cat (Järhult and Holst 1977 1978). The present experiments were performed to determine if somatostatin also eliminates this stimulatory effect on the glucagon release.

Methods. Four cats (mean weight 2.5 kg) were anesthetized with chloralose (50 mg/kg) and urethan (100 mg/kg) after induction with ether A tracheal cannula was inserted. The vagus nerves and the carotid arteries were dissected free in the neck. After heparinization, polyethylene catheters were placed in *the right femoral artery the portal vein and in the right axillary vein. The vagus nerves were cut in the* neck and about 30 min later a L infusion of synthetic cyclic somatostatin (Serono 50 μg/h) was started After 30 min of somatostatin infusion, the carotid baroreceptors were unloaded for 45 min by clamping the bilateral common carotid arteries. Portal blood samples for determination of immunoreactive glucagon and insulin and arterial blood samples for determination of glucose were drawn at intervals before and during the somatostatin infusion. Portal plasma glucagon concentration was determined by means of a recently described radioimmunoassay technique (Holst *et al* 1976), using an antiserum which *is highly specific for pancreatic glucagon.* Portal plasma insulin concentration was measured according to Albano *et al* (1972). Arterial plasma glucose concentration was measured with the glucose-oxidase method.

Results Fig. 1 illustrates that somatostatin lowered the basal glucagon and insulin levels in portal plasma. However somatostatin could not prevent a large increase occurring in the portal plasma glucagon concentration (IRG) in response to the baroreceptor unloading (= carotid occlusion) IRG thus rapidly rose from 102 ± 31 pg/ml to 256 ± 43 pg/ml in response to carotid occlusion and this level was roughly maintained until the clamps were removed, when IRG *promptly* returned to its pre-occlusion level Carotid occlusion could not further depress the somatostatin-induced lowering of the portal plasma insulin concentration (IRI). Somatostatin did not change the basal arterial plasma glucose concentration, which rose by about 4 mM *upon carotid occlusion.*

Fig. 2 shows a comparison between the changes of IRG in response to the unloading of the carotid baroreceptors with or without somatostatin infusion. Panel A illustrates that

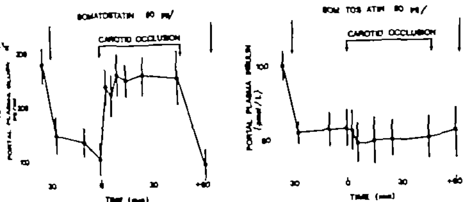


Fig. 1. Changes of portal immuno-reactive glucagon (left) and of insulin concentrations (right) in response to unloading of the carotid baroreceptors (= carotid occlusion) during somatostatin infusion in 4 cats. Mean values ± S.E. are given.

carotid occlusion led to a more pronounced increase in IRG in normal cats than in somatostatin-treated cats. It should be noted, however that the pre-stimulatory glucagon concentration was 3 times higher in the intact cats than in the somatostatin treated cats and the same changes in IRG are calculated in percentage, there was no statistically significant difference between the two groups of animals (Panel B).

Comments. Several previous studies have demonstrated that somatostatin lowers the basal plasma glucagon level and that it blocks the stimulatory action of different secretagogues on the A-cell. These findings have supported the hypothesis that somatostatin may interfere with a late step in the hormone-releasing mechanism common for all secretagogues, e.g. the calcium dynamics. However the present results suggest that glucagon might be released by some stimulus which is not completely inhibited by somatostatin. The exact mechanism(s) by which glucagon is released in response to baroreceptor unloading is not known, but the reflex is at least partly dependent on the sympatho-adrenal system, since the hyperglucagonemia is substantially reduced after cutting the splanchnic nerves (Järhult and Holst 1978). An interference by somatostatin with adrenergic mechanisms has been suggested by Smith *et al.* (1977) who found that the somatostatin-mediated inhibition of basal glucagon release was prevented by blockade of the α-adrenoceptors. A link between somatostatin and the sympatho-adrenal system with regard to endocrine pancreas is also supported by Järhult and Lundquist who observed that somatostatin can be released from the cat's pancreas in response to sympathetic stimulation (unpublished results).

The dose of somatostatin which has been used in this study (20-25 μg/kg/h) is somewhat higher than doses which have earlier been found to effectively suppress the glucagon release. For instance, Gerich *et al.* (1974) found that 20 μg/kg/h of somatostatin completely abolished the arginine-induced glucagon secretion in man. We therefore consider it unlikely that the increase in IRG upon carotid occlusion could be caused by too low a dose of somatostatin. Furthermore, the dose was sufficient for a marked reduction of the basal glucagon and insulin levels. It is therefore suggested that the glucagon release in response to baroreceptor unloading might be partly linked to some, yet unidentified, somatostatin-insensitive releasing mechanism.

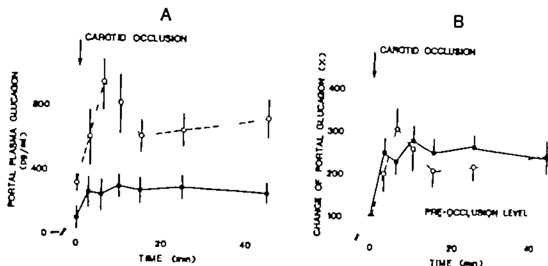


Fig. 2. Changes of portal plasma immuno-reactive glucagon concentration during unloading of carotid baroreceptors in cats during (●—●) or without (○—○) somatostatin infusion in cats. Results from intact cats have been calculated from previous published data (Järhult and Holst 1978). The changes in absolute terms are presented in Panel A, whereas Panel B depicts the changes in percent of the pre-occlusion value. Mean values ± S.E. are given.

This work was supported by grants from The Swedish Medical Research Council (B 78-04X 2210-12A) and from Maggie Stephen's Foundation.

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er Communications:

HELLSTRAND, P. Effects of hypoxia on the rat portal vein *in vitro*. P gradients in tissue and surrounding fluid

SCHULTZBERG, M., T. HÖKFELT, J. M. LUNDBERG, L. TERENIUS, L.-G. ELFVIN and R. ELDE. Enkephalin-like immunoreactivity in nerve terminals in sympathetic ganglia and adrenal medulla and in adrenal medullary gland cells

JÄRHULT, J., B. AHRÉN, M. ERICSSON and J. HOLST. Failure of somatostatin to eliminate the glucagon release induced by baroreceptor unloading in the cat

Supplement appended.

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The following symbols and units, recommended by the SI, are being used in Acta Physiologica Certain units, not included in SI, will still be permitted.

SI units with recommended symbols

Units	*Symbols*
kilogramme	kg
second, millisecond	s ms
mole, millimole, micromole, nanomole, picomole	mol mmol µmol nmol pmol
meter millimeter micrometer nanometer	m mm µm nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N (kg m/s^2)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m^2)

Conversion factors to be given in Methods.

Permitted non-SI units

Units	*Symbols*
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg (1.333 bar)
(millibar)	mbar (100 Pa
curie	Ci
liter milliliter microliter	l ml µl
degree Celsius	°C